Genetic analysis of postzygotic hybridisation barriers in Arabidopsis thaliana

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Award date:
2010

Awarding institution:
University of Bath

Genetic analysis of postzygotic hybridisation barriers in *Arabidopsis thaliana*

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A thesis submitted for the degree of Doctor of philosophy

University of Bath

Department of Biology and Biochemistry

October 2010

Signed:

Ahmed Bolbol

For my Father

Acknowledgements

I would like to thank my supervisor Prof. Rod Scott for his never-ending patience, encouragement, generous support, helpful guidance and understanding during my PhD project. Many thanks for Dr. Melissa Spielman, who I'll never forget for managing the project with my supervisor and for her technical support and helpful comments on my first experiments. I am also very grateful to Dr. Sushma Tiwari for unending support, guidance, and for sharing her experience and wealth of knowledge of molecular biology. Many thanks also to Dr. James Doughty for his encouragement and helpful discussions and to Dr. Paula Kover for her contribution to the QTL mapping and for helpful comments. Thanks to my colleague Alec Topham for his assistance with computing. Thanks to all lab members 3S 1.13 for their support and friendship: Rhiannon Hughes, Lucy Mathers, Ahlam Bouariky, Lyndsay Dytham, Alec Topham, Holly Smith-Baedorf, Valentyna Varenko, Richard Mead, Baoxiu Qi, Sapu Pradeepika, and Lihua Zhang. Grateful acknowledgements are given to Angie Vale and Julia Watling for caring for my plants, and to all members and technical staff in the Biology and Biochemistry department for their kind cooperation.

I would like to thank the Egyptian government for funding and continuous care and support. Thanks also to all staff members and friends: Prof. Ahmed Ghareeb, Dr. Alaa Said, Waled, Mahmoud, Mostafa, and Nabih in the University of Zagazig, Egypt, for their support and encouragement.

Appreciation go to my Mum, Dad (God bless him), brother, and sisters and all other members of my family. I'll not forget my wife Eman for her patience and continuous support and my lovely children Toleen and Mohammed.

Abstract

Most studies of plant hybridisation are concerned with documenting its occurrence in different plant groups. Many flowering plants are polyploids and seeds developed from crosses between individuals of different ploidies usually show abnormal features and often abort. The success or failure of interploidy crosses is very important to understanding the evolution of plants as well as to agriculture, but much remains to be learned about the nature of hybridisation barriers. Several mechanisms have been proposed to explain postzygotic barriers, including negative interactions between diverged sequences, global genome rearrangements, and widespread epigenetic reprogramming. Some recent advances in our understanding of the process of hybridisation are derived from different experimental studies on a series of *A. thaliana* ecotypes. Crosses between diploid (2x) and tetraploid (4x) individuals of the same ecotype can result in F_1 lethality, and this dosage-sensitive incompatibility plays a major role in polyploidy speciation research. We have performed interploidy crosses between different diploid maternal *A. thaliana* ecotypes and tetraploid paternal Col-0 ecotype and identified a genetic variation in F_1 lethality. We also found that maternal parents of some ecotypes such as Tsu-1 suppressed the F_1 lethality caused by paternal-excess interploidy cross of Col-0 ecotype. A preliminary mapping exercise produced advanced backcross populations that are suitable for mapping maternal modifiers and for the identification of modifier genes. Furthermore, we studied the killer effect caused by Col-0 and identified three additive QTL that affect the rate of postzygotic lethality in F_1 during interploidy crosses. This information will facilitate the identification of paternal genes that cause F_1 lethality and contribute to reproductive isolation.

The *moa-1* (*mosaic aneuploidy 1*) mutant of *A. thaliana* was obtained in a screen of chemically (EMS) mutagenised seeds of Landsberg *erecta* (L*er*). *moa-1* has various phenotypic differences to wild type; the preliminary karyotype analysis showed that the cells of individual *moa-1* mutant plants have a variable number of chromosomes (usually between 11-18). In contrast, the cells of wild type *Arabidopsis* plants and conventional aneuploids have a fixed number of chromosomes in each somatic cell. This data showed that all *moa-1* plants have an abnormal number of chromosomes and thus they were termed as mosaic aneuploids.

Abbreviations and Acronyms

ANOVA	analysis of variance
A. thaliana	*Arabidopsis thaliana*
Bla	Blanes/Gerona
Bur	Burren
CIM	composite interval mapping
Col	Columbia
Co	Coimbra
CSS	chromosome substitution strains
Cvi	Cape Verdi Islands
DAP	days after pollination
DNA	deoxyribonucleic acid
EBN	endosperm balance number
EMS	ethylmethane sulphonate
er	erecta
Kas	Kashmir
L*er*	Landsberg erecta
LOD	log-likelihood
moa	mosaic aneuploidy
NASC	Nottingham Arabidopsis Stock Centre
Ob-0	Oberusel/Hasen
Per	Perm
QTL	quantitative trait loci
RILs	recombinant inbred lines
se	standard error of the mean
SRL	single recombinant line
STAIRS	stepped aligned inbred recombinant strains
Stw	Stobowa/Orel
TAIR	The Arabidopsis Information Resource
Ws	Wassilewskija (Vaseljevisi)/Dnjepr

Contents

Chapter 1

1. General introduction

Hybridisation can have several meanings for evolutionary biologists, but can be restricted to an organism that developed from a cross fertilisation between individuals of different species. Hybridisation is useful in transferring genetic variability in cultivated species, for example as a source of disease and pest resistance for crop improvement. The interspecific hybridisation process is accepted as a major mechanism for generating novelty in the plant kingdom (Abbott, 1992). Several evolutionary studies were performed on hybridisation during the early 20th century. Wingë (1917) showed that new and constant hybrid species could be derived by the duplication of a hybrid's chromosome complement (allopolyploidy). This hypothesis was confirmed experimentally in a variety of plant species. Work carried out by Müntzing (1930) on homoploid hybrids or recombinational speciation found that sorting of chromosomal rearrangements in later generation hybrids may lead to the formation of a new homozygous population. The new hybrid population may be fertile, stable, and similar to their parents in their ploidy level. Anderson (1936) suggested that the products of interspecific hybridisation, particularly those resulting from backcrossing or introgression, might be favoured by selection and thus contributes to adaptive evolution within population.

1.1. Hybridisation barriers in plants

Most studies of plant hybridisation are concerned with documenting its occurrence in different plant groups. However, understanding the basis of the process of hybridisation and of hybridisation barriers between species is important in understanding the process of speciation and the extent of gene flow between species (Rieseberg and Blackman, 2010). Barriers to hybridisation restrict gene flow between species and may play a major role in speciation (Dilkes *et al*., 2008; Rieseberg and Blackman, 2010). Recently, the genetic basis of these barriers to gene flow have been attributed to so called 'speciation genes' that can act at multiple prezygotic and postzygotic stages of the life-cycle (Figure 1.1; Rieseberg and Blackman, 2010). Arnold (1998) found that in angiosperms reproductive barriers could be divided into premating (pre-pollinating), post-pollinating, and post-fertilisation (postzygotic) types. Premating barriers include spatial, temporal, and mechanical barriers, as well as morphological specializations that affect pollinator

behavior (Grant, 1994; Vickery, 1995 and Lee *et al*., 2008). Post-pollination hybridisation barriers can be prezygotic or postzygotic (Figure 1.1). Typical prezygotic post-pollination barriers arise from pistil and pollen interactions that affect the rates of both pollen germination and pollen tube growth resulting to a preference for conspecific gametes (Howard, 1999). These differences in rates represent potential reproduction barriers (Lee *et al*., 2008). Postzygotic barriers can be subdivided into extrinsic (ecogeographical isolation, mechanical isolation, conspecific pollen precedence) and intrinsic types. This latter type is most directly relevant to the present study, since it includes hybrid inviability as well as hybrid sterility (Martin *et al*., 2005; Figure 1.1).

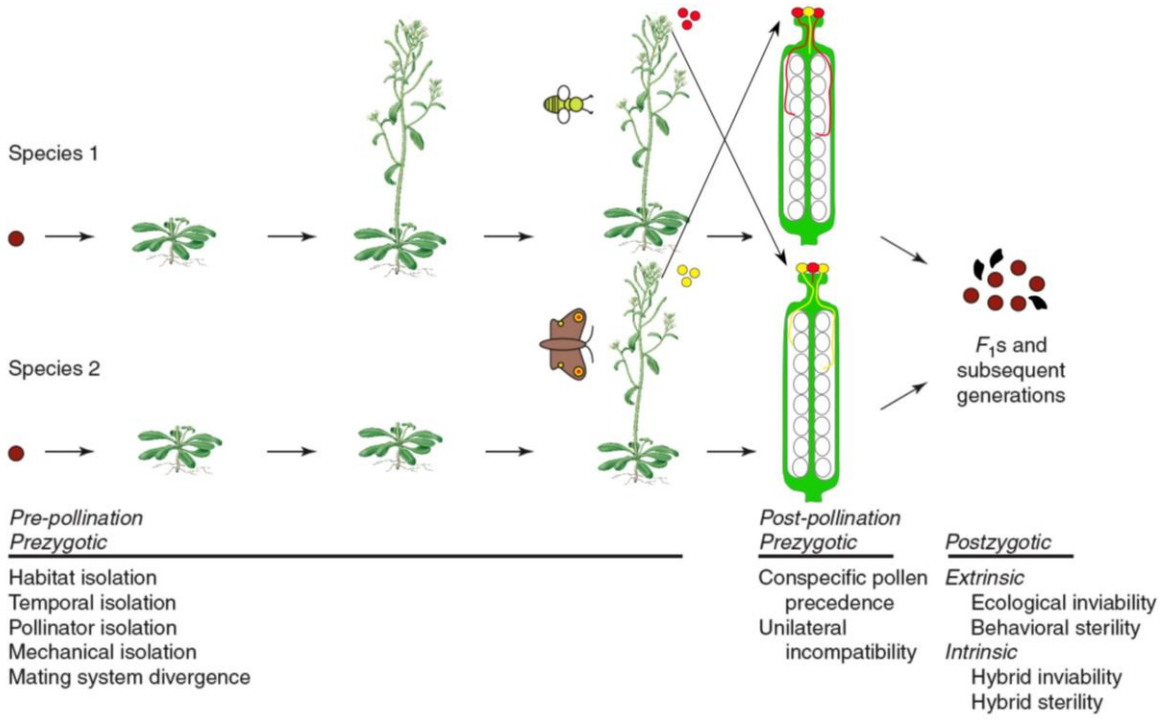

Figure 1.1: Reproductive isolating barriers in flowering plants (Modified from Rieseberg and Blackman, 2010).

1.1.1. Postzygotic hybridisation barriers in plants

Intrinsic postzygotic barriers are most frequently recognised via the production of inviable hybrid seed, and hybrid sterility or weakness within the immediate F_1 population and any subsequent generations (Stebbins, 1958; Coyne and Orr, 1998; Rieseberg and Carney, 1998; Tiffin *et al*., 2001, and Bushell *et al*., 2003). The earliest acting intrinsic

postzygotic barrier is due to the failure of seed development. For instance, Johnston *et al*., (1980) and Lin (1984) showed in their work with karyotypic variants and interspecies hybrids in plants that postzygotic hybridisation barriers occurs if the contributions of both pollen and seed parents deviate from the normal 2 maternal (m):1 paternal (p) ratio in the endosperm, indicating that a balance between these contributions is required for normal sexual reproduction.

The success or failure of crosses between related plant species has been the subject of many studies (Thompson, 1930; Haig and Westoby, 1991; Rieseberg and Carney, 1998; Tiffin *et al*., 2001) and the genetic mechanisms and the underlying speciation genes that govern reproductive barriers between species are now being elucidated (Rieseberg and Black, 2010; Table 1.1). Hybrid sterility or inviability (necrosis) has been studied in many species (Watkins, 1932; Stebbins, 1958; Wan *et al*., 1996; Li *et al*., 1997; Bomblies *et al*., 2007) and a variety of mechanisms have been proposed to explain the operation of this postzygotic barrier and the fitness problems of the hybrids such as negative interaction between diverged sequences, widespread epigenetic reprogramming, and rearrangements of the global genome (Rieseberg and Carney, 1998; Bushell *et al.,* 2003). Moreover, some genetic differences are proposed as the source of fitness problem to the hybrid from postzygotic barriers such as different chromosome arrangements or different alleles that are unable to function correctly together in the hybrid (Coyne and Orr, 1998). In addition, Burke and Arnold, (2001) found that two common mechanisms are thought to be the cause of postzygotic isolation; 1) The deleterious interactions between heterospecific genes may lead to hybrid sterility and hybrid inviability, 2) Chromosomal rearrangements in the parental species may result in abnormal meiotic products in their hybrids with negative effects on hybrid fertility. More recently, Bomblies and Weigel (2007) have suggested that disease resistance genes play an important role in reproductive isolation (Table 1.1). Strong supportive evidence has come from the discovery that incompatibilities among complementary disease resistance genes are the cause of necrosis in crosses between *A. thaliana* ecotypes (Bomblies *et al*., 2007). Similarly, disease resistance genes appear to mediate necrosis in crosses between rice varieties (Yamamoto *et al*., 2010).

Table 1.1 Genes that underlie intrinsic type postzygotic barriers.
(Modified from Rieseberg and Blackman, 2010)

Gene	Normal function	Organism	Level	Barrier phenotype	Likely genetic cause	Reference
Hybrid inviability						
Cf2 – an extracellular leucine-rich repeat receptor-like gene	Resistance to fungal pathogen	*Solanum lycopersicon*/ *S. pimpinellifolium*	Inter-specific	Hybrid necrosis	Gene copy number variation	Dixon et al., 1996; Kruger et al., 2002
RC3 – encodes an extracellular cysteine protease	Perception of fungal Avr proteins	*S. lycopersicon*/ *S. pimpinellifolium*	Inter-specific	Hybrid necrosis	Changes in protein sequence	Kruger et al., 2002; Rooney, 2005
DANGEROUS MIX 1	Disease resistance	*Arabidopsis thaliana*	Intra-specific	Hybrid necrosis	Gene copy number variation	Bomblies et al., 2007
HISTIDINOL-PHOSPHATE AMINO-TRANSFERASE (*HPA1* and *HPA2*)	Synthesis of essential amino acid, histidine	*A. thaliana*	Intra-specific	Arrest of hybrid seed development	Reciprocal silencing of duplicate genes	Bikard et al., 2009
TRANSPARENT TESTA GLABRA2 (*TTG2*) – a WRKY transcription factor	Regulates epidermal cell fate	*A. thaliana*	Intra-specific	Lethality of interploidal hybrids	Cis-regulatory mutations	Dilkes et al., 2008
HBD2 – encodes a casein kinase	Root development and hormone sensitivity	*Oryza sativa*	Inter-sub-specific	Hybrid necrosis	Change in protein sequence	Yamamoto et al., 2010
HWH1 – encodes a GMC oxidoreductase	Catalyses oxidation–reduction reactions	*O. sativa*	Inter-sub-specific	Hybrid necrosis	?	Jiang et al., 2008
Hybrid sterility						
S5 – encodes an aspartate protease	Disease resistance signalling and cell death	*O. sativa*	Inter-sub-specific	Hybrid female sterility (embryo sac sterility)	Changes in protein sequence	Chen et al., 2008
SaM – a SUMO E3 ligase-like gene	Post-translational modification	*O, sativa*	Inter-sub-specific	Hybrid male sterility (pollen abortion)	Substitution in intron-splicing site, leading to truncated protein	Long et al., 2008
SaF – encodes an F-box protein	Mediation of protein-protein interactions	*O. sativa*	Inter-sub-specific	Hybrid male sterility (pollen abortion)	Amino acid substitution	Long et al., 2008
mtRPL27 – Nuclear-encoded mitochondrial ribosomal	Translation of mitochondrial	*O. sativa* O. glumaepatula	Inter-specific	Hybrid male sterility (pollen abortion)	Reciprocal silencing of duplicate genes	Yamagata et al., 2010

Endosperm abortion resulting from endosperm breakdown is a common intrinsic postzygotic hybridisation barrier in plants (Lester and Kang, 1998; Datson *et al*., 2006). In interspecies crosses, endosperm abnormality and breakdown is often mentioned as the cause of seed abortion following successful fertilisation, with embryo abortion as a secondary effect (Watkins, 1932; Brink and Cooper, 1947; Stebbins, 1958; Haig; Westoby, 1991; Bushell *et al*., 2003).

Postzygotic lethality in interploidy crosses is hypothesized to play an important role in polyploid speciation by genetically isolating populations of divergent ploidy (Johnston *et al*., 1980; Burton and Husband, 2000; Dilkes *et al*., 2008). Successful hybridisation in interploidy crosses is rare. Postzygotic hybridisation barriers have been widely studied in many interploidy crosses within the same or different plant species. For example, Repkova *et al*., (2006) studied the impact of post-fertilisation barriers on embryo development in *Trifolium* spp. after interspecific crosses between diploid and tetraploid *T. pratense* and diploid wild species *T. alpestre* L., *T. medium* L. and *T. sarosiense* Hazsl. In the crosses between diploid *T. pretense* and the others the growth of the pollen tube was arrested. When *T. pretense* (4x) was crossed with *T. alpestre* or *T. sarosiense* no hybrid embryos could be detected by seed clearing followed by microscopy despite an enlargement of the immature seeds was observed. When *T. medium* was used as the pollen parent in a cross with *T. pretense* (4x) as the seed parent, different stages of embryos till torpedo stage were observed 7 days after pollination (DAP).

Also, Fu *et al*., (2009) found severe postzygotic crossability hybridisation barriers resulting in difficulties in transferring useful genes from *Oriza meyeriana* to *Oriza sativa* in their interspecific hybridisation crosses. They found that hybrid embryo abortion is a major reason for this hybridisation barrier, inducing no production of viable hybrid seeds in maternal plants. Additionally, postzygotic reproductive barriers in the intergeneric hybridisation between *Chrysanthemum grandiflorum* (Ramat.) Kitam. (female parent) and *Ajania przewalskii* Poljak (male parent) plants resulted in low yield of seeds as demonstrated by Deng *et al*., (2010).

Recently, several genes have been implicated in intrinsic postzygotic barriers that operate at the level of hybrid seed failure. For example, copies of the *HISTIDINOL-PHOSPHATE AMINO-TRANSFERASE* (*HPA1 and HPA2*) gene, which encodes a key enzyme in the biosynthesis of the essential amino-acid histidine, are subject to differential silencing in different ecotypes of *A. thaliana* resulting in a proportion of hybrid progeny

lacking functional *HPA* expression (Bikard *et al*., 2009; Table 1.1). Seeds containing such progeny undergo developmental arrest and the seed aborts. An extensive survey of *A. thaliana* ecotypes revealed an array of different mechanisms fort HPA silencing including deletions, early stop codons and/or loss of expression. This confirmed a long standing idea that the reciprocal silencing of duplicate genes is a potentially widespread cause of intrinsic postzygotic barriers (Werth and Windham, 1991).

Differential expression of the *TRANSPARENT TESTA GLABRA2* (*TTG2*) gene has also been implicated in seed lethality following crosses between *A. thaliana* ecotypes, in this instance, when the parental plants differ in ploidy level (Dilkes *et al*., 2008). Landsberg *erecta* (L*er*) and Columbia (Col-0) ecotypes vary in their tolerance to inter-ploidy crosses. Low resolution genetic analysis first identified a major QTL for this interploidy lethality, and then fine mapping revealed that a maternally expressed WRKY transcription factor, TTG2, was explained the QTL effects. Sequence and expression comparisons of the L*er* and Col *TTG2* alleles found cis-regulated differences in expression levels rather than changes in coding sequence were the likely cause of variability in inter-ploidy hybrid inviability.

1.2. The model plant *A. thaliana*

A. thaliana is a small flowering herb that is widely used as a model organism in plant biology. *Arabidopsis* belongs to the mustard family (Brassicaceae) and it is mainly autogamous with infrequent out crossing under laboratory condition. Although not of major agronomic significance, *Arabidopsis* offers important advantages for basic research in genetics and molecular biology (Griffing and Scholl, 1991). It was the first plant for which the complete genome was sequenced and has a small genome size (120 Mb) (Can *et al.* 2003). The genome is organized into five chromosomes having an estimated 20,000 genes (Meinke *et al.* 1998). The cultivation process of *A. thaliana* is very simple and the entire life cycle from seed to seed can be completed in 6 weeks.

Arabidopsis users benefit from the seed stock centers such as Nottingham Arabidopsis Stock Centre (NASC), from which large collection of naturally occurring ecotypes and mutant lines are available. *Arabidopsis* ecotypes show different variation in many physiological traits and provide a useful resource for identifying the molecular basis of complex traits exploiting the polymorphism in nucleotide sequences. The use of *A. thaliana* chromosome substitution lines (CSS) (Koumproglou *et al.,* 2002) and

recombinant inbred lines (RIL) such as (Col/L*er*) (Lister and Dean, 1993) can serve as powerful tools for genetic molecular mapping studies especially for agriculturally important traits such as yield, quality and some forms of disease resistances that are controlled by a number of genes known as quantitative trait loci (QTL). Since 2000, all information about *Arabidopsis* including genes, polymorphism, markers, sequences, maps, clones, and access to DNA and seed stocks (Garcia-Hernandez and Reiser, 2002) are available through the TAIR website (www.arabidopsis.org).

1.3. Endosperm development with special reference to *A. thaliana*

The development of seeds in higher plants is initiated by double fertilisation, a process in which the two haploid male gametes resulting from the division of the single generative cell of the pollen grain unite with cells of the megagametophyte. One gamete fuses with the haploid egg nucleus to produce the diploid embryo, whilst the other gamete fuses with two haploid polar nuclei of the central cell to form a triploid product that develops into the endosperm (Figure 1.2); (reviewed by Haig, 1990; Brown *et al*., 1999).

A. thaliana has been used as a model plant for studying the development of the endosperm. After fertilisation, the development of the endosperm starts by successive divisions of the triploid primary endosperm nucleus without cytokinesis (Mansfield and Briarty, 1990b; Berger, 1999, 2003). During the first few free-nuclear divisions, some nuclei migrate to the micropylar and chalazal poles (Mansfield and Briarty, 1990a; Brown *et al*., 1999; Herr, 1999) forming three distinct mitotic domains of the endosperm, as verified by time-lapse observations (Boisnard-Lorig *et al*., 2001).

In wild type seeds, cellularisation of the endosperm is coupled to the eighth cycle of synchronous mitosis in the peripheral endosperm that are initiated in the micropylar mitotic domain and spread as a wave across the peripheral endosperm. At the chalazal pole, a prominent multinucleate structure known as the chalazal endosperm is formed (Scott *et al*., 1998a). The embryo sac expands progressively, increasing seed volume, as the peripheral endosperm proliferates. At the time of endosperm cellularisation, the embryo is usually at the heart stage of development. After cellularisation, the embryo maintains its development and absorbs and assimilates the contents of the endosperm cells. Only a single aleurone layer of endosperm cells persists in the mature seed.

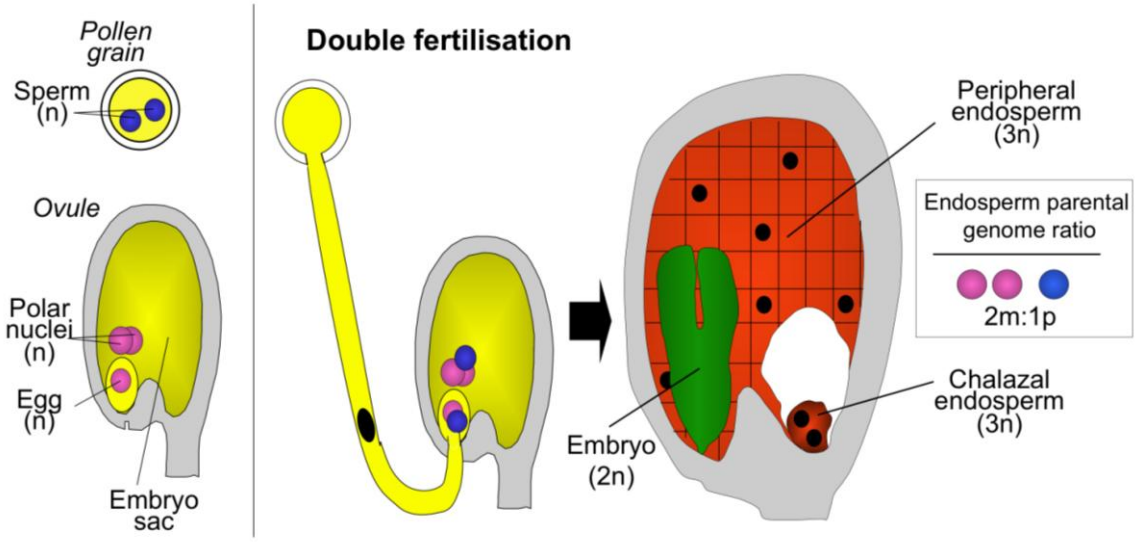

Figure 1.2: Double fertilisation in *A. thaliana.*
Double fertilisation results in two fertilisation products, the endosperm and embryo. The haploid egg is fertilised by a sperm nucleus and gives rise to a diploid embryo. Two haploid polar nuclei in the central cell fuse with the second sperm and this process results in the triploid primary endosperm nucleus. This nucleus replicates and divides to form the endosperm.

1.3.1. Consequences of parental imbalance in interploidy/interspecific crosses

In some plants such as *Zea mays* and *A. thaliana*, reciprocal interploidy crosses have established that the effect of parental ploidy on endosperm development is directional. For instance, in *A. thaliana* both [2x X 4x] and [4x X 2x] interploidy crosses showed parent-of-origin dependent effects on seed development. The maternal excess cross produced relatively small seeds, whereas large seeds resulted from the paternal excess cross (Scott *et al*., 1998a). In maize, the ratio between paternal and maternal contributions in the endosperm is important and any change from a balanced [2x X 2x] cross results in kernel abortion (Cooper, 1951; Li and Dickinson, 2010). Lin (1982; 1984) showed that a specific ratio of parental ploidies was necessary using the *intermediate gametophyte* (*ig*) mutation that conditions the production of a variable number of polar nuclei within a single embryo sac. The *ig* mutant was used as a seed parent in crosses to wild type diploid or tetraploid pollen parents to produce endosperm with an altered parental genome ratio and ploidy varying from 2x to 9x. These crosses generated seeds with 2x or 3x embryos that were associated with a range of endosperm karyotypes that

differed in total ploidy and maternal and paternal genomic ratios. Lin found that endosperms that deviated from a 2 maternal (m):1 paternal (p) ratio were not able to complete development and were associated with seed abortion. This led Lin to conclude that a 2m:1p genomic ratio rather than a specific ploidy is the critical factor for normal endosperm development.

Unlike maize, in which crosses between a 2x and a 4x are lethal, *A. thaliana* tolerates some deviation from the 2m:1p genome ratio in the endosperm: *A. thaliana* seeds from [2x X 4x] crosses with a ratio of 2m:2p are viable, as are seed from [4x X 2x] crosses with a ratio of 4m:1p. Extra paternal genomes in the endosperm (paternal excess), for example in a [2x X 4x] cross, result in large seeds in which both the peripheral and chalazae endosperm proliferate to a greater extent than the balanced [2x X 2x] cross and endosperm cellularisation is delayed. Extra maternal genomes in the endosperm of the reciprocal cross (maternal excess) in a [4x X 2x] cross, produce small seeds following early cellularisation of an underproliferated endosperm (Scott *et al*., 1998a). Despite the development of abnormal endosperm both interploidy crosses result in the production of viable seed. This is in contrast to the outcome in many other species of crosses between 2x and 4x plants which result in seed abortion (Haig and Westoby, 1991).

The observed endosperm abnormalities in crosses between 2x and 4x *A. thaliana* are more severe in [2x X 6x] and [6x X 2x] crosses. These interploidy crosses resulted in endosperm with extreme paternal or maternal excess phenotypes and eventually seed abortion (Scott *et al*., 1998a). The interploidy cross [2x X 6x] resulted in the formation of seed that contained severely over-proliferated endosperm that failed to undergo cellularisation. Such seed contained an embryo arrested at the globular to heart stage and almost never germinated. The reciprocal interploidy cross [6x X 2x] produced inviable seed that were small, contained a small peripheral endosperm that cellularised very early. A similar outcome to extreme imbalance is observed in other *Arabidopsis* species. Comai found that seeds developed from crosses between [2x *A. thaliana* X 4x *A. arenosa*] enlarged at the normal rate for 10 days, but embryos within these seeds arrested at the globular stage. Such seeds subsequently collapsed and failed to germinate. However, in contrast [4x *A. thaliana* X 4x *A. arenosa*] crosses produced seed with embryos at a range of different stage; whilst most of this seed also aborted, a small proportion matured to viable seed (Comai *et al*., 2000). Bushell *et al*., (2003) consolidated this idea by showing that the cross between [2x *A. thaliana* X 4x *A. arenosa*] generated severely paternalised endosperm similar to those observed in [2x X 6x] crosses in *A. thaliana* (Scott *et al*.,

1998a). In contrast, they found that seeds developed from the [4x *A. thaliana* X 4x *A. arenosa*] cross fell into two categories: 60% were relatively large and plump and 40% were relatively small and shriveled. Their hypothesis was that that imprinting is involved in the hybridisation barrier between *A. thaliana* and *A. arenosa* supported by their observation that doubling the maternal ploidy, to make the [4x *A. thaliana* X 4x *A. arenosa*] cross, resulted in a high proportion of viable seed. However, endosperm in this cross exhibited a dramatic reduction in peripheral endosperm proliferation and restoration of endosperm cellularisation in comparison to [2x *A. thaliana* X 4x *A. arenosa*]. These crosses were interpreted as demonstrating the higher 'genomic strength' of *A. arenosa* relative to *A. thaliana*. Bushell *et al*., (2003) also concluded that the ability of the *A. arenosa* and *A. thaliana* genomes to function together to produce viable embryos in the [4x *A. thaliana* X 4x *A. arenosa*] cross and subsequently a healthy fertile hybrid suggests that there is no general incompatibility or incongruity between the two parental genomes due to sequence divergence.

1.3.2. The endosperm balance number (EBN) hypothesis

In interspecific or interploidy crosses, the success of seed development does not depend solely on the 2m:1p ratio in the endosperm. The varied outcomes from both crosses in several genera have lead to the development of several hypotheses to explain the basis for normal seed development following both intra and interspecific crosses. Early hypotheses suggested a need for a particular ratio of chromosome sets between the fundamental parts of the developing seed (i.e. maternal tissue, embryo and endosperm) for normal seed growth (Haig and Westoby, 1991). With the advent of the endosperm balance number (EBN) concept by Johnston *et al*., (1980) which requires 2m: 1p EBN balance ratio in the endosperm, a new understanding of both interecotype and interspecific crossability barriers has been recognized. According to the EBN hypothesis, each species has a genome-specific effective ploidy level which does not necessarily correspond to the actual ploidy. For example, the EBN has been used to explain the behavior of crosses within *Solanum* species (Johnston and Hanneman, 1980). These authors chose *Solanum chalconase* (2n=24) as the standard species and assigned this an EBN of 2. Other species were assigned an EBN value based on the success or failure of crosses to *Solanum chalconase*. Crosses between species with different EBN values generally resulted in failure of seed development whilst crosses between species with the same EBN value were

viable. Significantly, success or failure was not dependent on achieving a 2m: 1p genomic ratio within the endosperm.

There are some exceptions where modest deviations from the 2 maternal: 1 paternal ratio is tolerated. For instance, Ehlenfeldt and Hanneman, (1988) found that an excess of maternal EBN dose is tolerated better than paternal excess in crosses between 2EBN and 1.5EBN species. Whilst a [2 EBN X 1.5 EBN] cross produced small and viable seed, as similar to those observed in an *A. thaliana* [4x X 2x] cross, the reciprocal cross resulted in shriveled non-viable seed.

The EBN hypothesis has been extended to explain hybridisation in a number of other genera, implying that the EBN system is appropriate to a wide range of species (Carputo *et al*., 1999). Even though the term endosperm balance number (EBN) was proposed to explain the pattern of dosage-dependent incompatibility intrinsic to each species or ecotype (Johnston *et al*., 1980; Dilkes *et al*., 2008), such phenomena are not limited to angiosperms. For example, interspecies hybridisations in some vertebrates such as crosses within the genus *Hyla* showed increased fertility when the maternal parent genomes increased compared to the paternal one (Mable and Bogart, 1995).

EBN has been assigned to a large number of species (Johnston and Hanneman, 1982; Hawkes and Jackson, 1992). It is suggested that a change in regulation of the genes involved in EBN can lead to changes in overall EBN level, and can therefore lead to speciation (Ehlenfeldt and Hanneman, 1988 and Bushell *et al*., 2003). The EBN system enables predictions to be made regarding the outcome of crosses within or between species. For example, the EBN hypothesis was developed primarily to explain crosses between *Solanum* (potato) species (Johnston and Hanneman, 1982; Bamberg, 1994; Masuelli and Camadro, 1997) and later extended to include other genera such as *Lycopersicon* (tomato), *Datura* and *Trifolium* (clover) (Parrott and Smith, 1986; Ehlenfeldt and Hanneman, 1992; Johnston and Hanneman, 1999; Repkova *et al*., 2006).

1.4. Polyploidy: causes and consequences

Polyploidy has played a major role in evolution of flowering plants (Ramsey and Schemske, 1998; Soltis *et al*., 2003). Polyploidy is the presence of more than two sets of chromosomes within a nucleus, and it is widespread among plants. Moreover, there is strong evidence to suggest that 60-70 % of the flowering plants have polyploid ancestory (Wang *et al*., 2004; Köhler *et al*., 2010).

A. thaliana is an attractive model plant to demonstrate fundamental biological processes associated with evolutionary consequences and molecular mechanisms of polyploid formation. The small genome of *A. thaliana* may have been derived from three rounds of polyploidisation (Bowers *et al*., 2003, and Soltis, 2005). The combination of two genomes may create interactions that give rise to novel gene expression. As a result, polyploid species often display new traits and genetic variability (Wang *et al*., 2004).

Allopolyploids have multiple chromosome sets are formed through the combined processes of interspecific hybridisation and chromosome doubling (Otto and Whitton, 2000, and Soltis, 2005). In contrast, autopolyploids arise within a single species through chromosome multiplication (Otto and Whitton, 2000; Soltis, 2005, and Köhler *et al*., 2010).

Current studies of polyploid species have revealed genetic variability among and within populations, due to varied contributions of multiple parental individuals. Additionally, crossing among genetically different individuals of separate origin may generate greater genetic diversity through independent assortment. Furthermore, the result may be many genetically different polyploid individuals that may respond differently to various selection pressures, providing more opportunities for polyploidy species to survive changing environments (Soltis, 2005). Until now, the mechanisms of polyploid formation have not been entirely elucidated.

1.4.1. Triploid block theory and the formation of unreduced gametes

Triploidy occurs frequently in diploid species of both animals and plants as a result from the accidental fusion of 2n gamete to a regular 1n gamete. Errors during meiosis are likely a common cause of unreduced gametes in diploid individuals (d'Erfurth *et al*., 2008; Köhler *et al*., 2010). Triploids can also arise as a result of crosses between diploid and tetraploid individuals (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998).

In humans, triploidy is not tolerated and is implicated in postzygotic lethality (miscarriage) in approximately 2% of all conceptions between parents of apparently normal karyotypes (Menasha *et al.*, 2005). In contrast, some triploid fish and amphibia can grow to adulthood and some species are even fixed in the triploid state (Tock *et al*., 2002). Muller (1925) argued that triploidy is rarer in animals than plants because animals have a more complex development, with more organ systems that are more sensitive to gene dosage.

In plants, where crosses between diploid and tetraploid individuals of the same or related species, result in a frequent failure of seed set, a reproductive barrier known as the 'triploid block' is held responsible. The name is derived from the failure to produce the expected triploid progeny. The phenomenon of triploid block has been extensively studied within a variety of different species including brassica (Howard, 1939; Hakansson, 1956), *Lycopersicon* (Cooper and Brink, 1945), rye (Hakansson and Ellerstrom, 1950), maize (Cooper, 1951), *Galeopsis pubscens* (Hakansson, 1952), barley (Hakansson, 1953), *Primula* (Woodell and Valentine, 1961), and *Poinsettia* (Milbocke and Sink, 1969).

In plants most triploid embryos apparently die because of abnormal endosperm development (Satina and Blakeslee, 1938; Markes, 1966). Triploid block can result in a high degree of instant postzygotic reproductive isolation between tetraploids and their diploid progenitors, since nonviable progeny are formed by backcrossing to either parent (Ramsey and Schemske, 1998; Köhler *et al*., 2010).

In *A. thaliana,* crosses between diploids and tetraploids of the C24 and L*er* ecotypes did not exhibit the high levels of triploid block found in other species. However, Redei (1964) had earlier described a radically different outcome for reciprocal crosses between a diploid genotype 'W' and its autotetraploid 'K-16'. Whilst, the [K-16 (4x) X W (2x)] produced mainly (91.6 %) plump seed, the reciprocal [W (2x) and K-16 (4x)] cross produced mainly (97.3 %) shrivelled seed. The 'W' genotype used in the Redei is believed to be the Col-0 ecotype (pers comm. between George Redei (University of Columbia-USA) and Rod Scott (University of Bath-UK). Further strong evidence for this association was provided by Dilkes *et al*., (2008), who demonstrated essentially the same outcome using a *bona fide* Col-0 diploid and derived autotetraploid in reciprocal crosses. Dilkes *et al*., (2008) thus confirmed the existence of genetic variation for triploid block in *A. thaliana*, albeit of an unusual character i.e. asymmetric (paternal-only) triploid block.

Until recently, neither the formation of unreduced gametes nor the mechansim of triploid block was understood at the molecular level. The genetic basis of unreduced gamete production has been subjected to analysis in *A. thaliana* (Ramsey and Schemske, 1998 and Köhler *et al*., 2010). Unreduced male gametes are formed at high frequency in the *Arabidopsis parallel spindle 1* (*Atps1*) mutant by a parallel orientation of the spindle during meiosis II causing failure of the homologous chromosomes to migrate to opposing pole (d'Erfurth *et al*., 2008). On the other hand, the unreduced male gametes formed in the *jason* (*jas*) mutant are formed due to a failure in homologous chromosome pairing during meiosis I (Erilova *et al*., 2009).

Unreduced female gametes have been explained by many researchers; typical examples being the *switch 1/dyad* mutant formed by mitotic-like division in the female meiocyte at meiosis I (Mercier *et al*., 2001; Agashe *et al*., 2002). Omission of the second meiotic division (Bretagnolle and Thompson, 1995), which occurs in the *Arabidopsis omission of second division 1* (*osd1*) mutant also produces unreduced female gametes (d'Erfurth *et al*., 2009).

1.4.2. Aneuploidy and mosaic aneuploidy

Chromosome segregation is an important process in mitosis and must be performed correctly to ensure that the two resulting daughter cells have the same DNA content. Missegregation of chromosomes results in aneuploidy, something that is frequently found in cancers, suggesting that the machinery surveying the chromosome segregation process has somehow been compromised during the development of these tumours. Aneuploidy introduces dosage imbalance on a chromosomal scale and is proposed to alter phenotypes by simultaneously disrupting the stoichiometry of all dosage-sensitive gene products encoded by a chromosome, or subset of chromosomes (Birchler *et al*., 2001). Aneuploidy has played an important role in the study of plant genetics. Plants with aneuploid chromosome numbers were first reported in the hybrid between *Oenothera lata* and *Oenothera gigas* generations by Lutz (1909). However, the term aneuploidy was first introduced by Tackholm (1922) following her research in the genus *Rosa*. There are two types of aneuploidy usually observed 1) monosomy (2n-1) which is the lack of one of a pair of chromosomes, 2) trisomy (2n+1) that refers to an individual having additional chromosome to the normal diploid number (Hartwell *et al.* , 2004). Among aneuploids, trisomics by far are the best documented (Khush, 1973). Triploids were used as sources of trisomics in different plants such *Datura* (Blakeslee, 1921), maize (McClintock, 1929), etc; however, these studies introduced the idea that triploids could produce viable aneuploids, which were mostly trisomics.

Aneuploidy is often associated with distinct and usually deleterious phenotypes that are specific to the type of aneuploidy (Blakeslee, 1921; Khush, 1973; Henry *et al*., 2006). Henry *et al.*, (2005) stated that chromosome doubling of a plant usually has little effect on fertility, while drastic changes to the phenotype may results from adding a single chromosome and mainly could lead to sterility. In polyploid backgrounds, relative chromosome imbalance is reduced due to the overall higher chromosome copy number and aneuploidy can have minor phenotypic consequences (Ramsey and Schemske, 1998).

When aneuploidy is detected in a fraction of cells in an individual, it is called chromosomal mosaicism. Mosaic aneuploidy or the term "*mosaicism*" describes a situation in which different cells in the same individual have different numbers or arrangements of chromosomes (Montalenti, 1978). Youssoufian and Pyeritz, (2002) stated that aneuploid mosaicism is the presence of more than one genetically (karyotypically) distinct cell line within a single organism. Mosaic aneuploidy is frequently seen within human preimplantation embryos (Delhanty *et al*., 1993) and has been suggested to represent a common condition in humans (Voullaire *et al*., 2000). Until now the mosaic aneuploidy is not reported in plants; however, our preliminary data suggested that *A. thaliana* mutant plant *mosaic aneuploidy-1* (*moa-1*) is a mosaic aneuploid. Identifying the gene underlying this mutation would provide valuable insights into the mechanism of mosaic aneuploidy.

1.5. QTL genetic mapping

The anomalous behavior of the Col-0 [2x X 4x] crosses showing high F_1 lethality similar to those observed in crop species has opened up avenues to understand the basis of seed abortion following crosses. However, a single phenotypic trait could be determined by many genes that constitute quantitative trait loci (QTL). QTL mapping is the statistical study of the alleles that occur in a locus and the phenotypes that they produce. Because most traits of interest are controlled by more than one gene, it is important to study the entire locus of related genes to understand what might affect the genotype of an individual. In simple terms, QTL analysis is based on the principle of detecting an association between phenotype and genotype. Genetic mapping or linkage mapping refers to the determination of relative position and distances between markers along chromosomes. Genetic mapping is based on the principle that genes segregate via chromosome recombination during sexual reproduction (i.e. meiosis division), thus allowing their analysis in the progeny (Paterson, 1996).

Many agriculturally important characters such as yield, quality, and some forms of disease resistance are controlled by many different genes or quantitative traits (polygenic inheritance). The regions within genomes that contain genes associated with a particular quantitative trait art known as quantitative trait loci (QTL) (Collard *et al*., 2005). They can be attributed to the interactions between two or more genes and their environment. Nevertheless, QTLs can be molecularly identified to help map regions of the genome that contain genes involved in specifying a quantitative trait (Collard *et al*., 2005). This can be

an early step in identifying and sequencing these genes. QTL analysis to identify genomic regions associated with traits is known as QTL mapping (Paterson, 1996 and Mohan *et al*., 1997). In many biological researches, the QTL identification is accomplished by studying DNA markers. However, the use of DNA markers in plant breeding has opened a new area in agriculture called molecular breeding (Rafalski and Tingey, 1993). DNA markers that are linked to agronomically important genes may be used as molecular tools for marker assisted selection in plant breeding (Ribaut and Hoisington, 1998).

DNA markers arise from different classes of DNA mutations such as point mutations, rearrangements (insertion or deletion) or errors in replication of repeated DNA (Paterson, 1996). DNA markers are divided into three categories based on the method of their detection: hybridisation-based, polymerase chain reaction or PCR-based, and DNA sequence-based (Jones *et al*., 1997). The DNA markers are useful if they reveal differences between individuals of the same or different species (polymorphic markers). However, polymorphic markers are either dominant or co-dominant markers, thus both markers can easily differentiate between heterozygous and homozygous individuals. Additionally, the co-dominant markers indicates differences in size whereas dominant markers are either present or absent (Collard *et al*., 2005). A number of excellent reviews have been written about the construction of linkage maps, QTL analysis and the application of markers in marker assisted-selection such as (Tanksley, 1993; Jones *et al*., 1997; Collard *et al*., 2005; Semagn *et al*., 2006).

QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers. Markers are used to partition the mapping population into different genotypic groups based on the presence or absence of a particular marker locus and to determine whether significant differences exist between groups with respect to the trait being measured (Tanksley, 1993). Most QTL experiments to identify different phenotypic traits and marker genotypes have been carried out using several crossing methods from inbred lines including backcross (BC), advanced backcross, F_2, recombinant inbred (RI) populations, intermated recombinant inbred (IRI) populations, advanced intercross (AI) populations, advanced backcross populations, double haploid (DH) populations (Winkler *et al.*, 2003; Broman, 2005; Kao, 2006).

The use of *A. thaliana* recombinant inbred lines (RILs) can serve as powerful tools for genetic mapping studies in plants (Lister and Dean, 1992). Several RIL populations have been widely used for mapping in different organisms including mice (Bailey, 1981; Carnerio *et al*., 2009), maize (Burr *et al.*, 1988; Austin and Lee, 1996), rice (Nair *et al*.,

1995; Cho *et al*., 2010), sunflower (Langar *et al.*, 2003), soybean (Mansur *et al*., 1996; Choi *et al*., 2010), and *Arabidopsis thaliana* (Kobayashi and Koyama, 2002; Kobayashi *et al.*, 2004; Lisec *et al*., 2009). However, there are many useful *A. thaliana* QTL studies that investigated life history traits such as flowering time (Clarke *et al*., 1995; Alonso-Blanco *et al.*, 1998), seed dormancy (van Schaar *et al*., 1997), growth (Mitchell-Olds, 1996; Alonso-Blanco *et al.*, 1999), and seed size (Alonso-Blanco *et al.*, 1999).

1.6. Research aim and objectives

The aim of this research project was to investigate the role of hybridisation barriers in seed development. Our research aim was carried out in two main aspects:

1) To determine the molecular genetic basis of mosaic aneuploidy in *A. thaliana.*

2) To understand the molecular genetic basis of the *A. thaliana* postzygotic hybridisation barrier.

The objectives of this research were as follows:

1. Determine the molecular genetic basis of the mosaic aneuploidy phenotype of the *moa-1* mutation. The approach was to 1) confirm the mosaic aneuploidy phenotype by extensive karyotype analysis; 2) study the genetics of *moa-1* inheritance; 3) attempt to map and identify the *moa-1* gene.

2. Determine the extent of genetic variation for triploid block in *A. thaliana.*

Dilkes *et al*., (2008) working with the Col-0 ecotype first demonstrated the existence of genetic variation for triploid block in *A. thaliana.* In Col-0, in contrast to L*er* and C24, the [2x X 4x] cross exhibited high levels of triploid block; the reciprocal [4x X 2x] cross behaved as L*er* and C24 in being non-abortive. An important question therefore was which of the two behaviors - no triploid block or paternal-only triploid block - or some other permutation, is most representative of the *A. thaliana* species. The experimental approach was to test the crossing behaviour of multiple additional ecotypes and to use meiotic mutants to investigate aspects of the Col paternal-only triploid block.

3. To identify and characterise a potent maternal modifier that prevents expression of Col-0 triploid block. This was expected to provide valuable insights into the process of Col-0 induced seed abortion, particularly at the cell biological and molecular genetic level.

4. Determine the genetics of the Col-0 killing trait.

The approach taken to increasing our understanding of the mechanism underlying the Col-killer trait was to map and ultimately identify the gene or genes responsible.

Chapter 2

2. Materials and Methods

2.1. Materials

2.1.1. Plant material

Seed stocks of Col-0 (N1093), Col 4x, L*er* 2x (NW20), and L*er* 4x ecotypes were kindly donated by L. Comai (UC Davis, USA). RLD 2x seeds were kindly donated by B. Fisher (University of Berkeley, USA). The ecotypes C24 (N906), Tsu-0 (N1564), Tsu-1 (N1640), Cvi-0 (N1096), Kas-1 (N1264), Bur-0 (N1028), Per-1 (N1444), Stw-0 (N1538), Co-2 (N1086), Ob-0 (N1418), Ws-0 (N1602), and Bla-0 (N970) were obtained from the Nottingham Arabidopsis Stock Centre (NASC, UK). Tetraploids of the above mentioned accessions were generated in our laboratory by Prof. Rod Scott (University of Bath, UK). The *moa* mutant was found in a screen for big seeds by H. Godwin (A student in the Scott Lab, University of Bath). The *Atps1-1* mutant seed were a gift from R. Mercier (INRA, Versailles, France). *Jas-3* mutant seed were from K. Köhler (Department of Biology and Zurich-Basal Plant Science Centre, Switzerland). The Chromosome Substitution Strains (CSS) (Table 2.1) were kindly donated by M. Kearsey (University of Birmingham, UK). The Stepped Aligned Inbred Recombinant Strains (STAIRS) SRL1 (N9431), L*er* interval 0-84 cM in chromosome 1, the Recombinant Inbred Lines (RILs), Lister and Dean Col and L*er* background, were obtained from NASC (Table 2.2).

Table 2.1: Chromosome substitution lines (CSS) used in this research study.

CSS	Stock number	Description
2	N9433	Col-0 chromosome 2
3	N9434	replaced
4	N9435	by
5	N9436	L*er* chromosome 2

Table 2.2: Information on the Lister and Dean (Col/L*er*) recombinant inbred lines (RIL) used in this research study.

RIL	Stock number	RIL	Stock number	RIL	Stock number
1	N1900	24	N1936	47	N1968
2	N1901	25	N1937	48	N1969
3	N1903	26	N1938	49	N1970
4	N1904	27	N1940	50	N1971
5	N1906	28	N1943	51	N1972
6	N1907	29	N1944	52	N1974
7	N1909	30	N1945	53	N1977
8	N1910	31	N1946	54	N1978
9	N1911	32	N1948	55	N1979
10	N1912	33	N1949	56	N1980
11	N1914	34	N1950	57	N1982
12	N1915	35	N1951	58	N1983
13	N1916	36	N1952	59	N1984
14	N1917	37	N1953	60	N1985
15	N1918	38	N1954	61	N1988
16	N1919	39	N1955	62	N1989
17	N1921	40	N1956	63	N1990
18	N1923	41	N1957	64	N1991
19	N1925	42	N1958	65	N1992
20	N1926	43	N1960	66	N1993
21	N1929	44	N1961	67	N1997
22	N1930	45	N1963	68	N1998
23	N1931	46	N1966	69	N1999
24	N1933	47	N1967	70	N04686

2.1.2. Primer design and synthesis

Primer pairs for genotyping were designed using Primer 3.0 (http://frodo.wi.mit.edu/primer3/) and they were analysed for melting temperature and secondary structure using NetPrimer (http://www.premierbiosoft.com/netprimer/index.html). Primer synthesis was done by Invitrogen. Further information about individual primer pairs are provided in Table 2.3.

Table 2.3: Primer sequences used for genotyping and expression analysis

Marker name	Primers, 1=forward, 2= reverse	Annealing Tm (°C)
BKN000001028	1. TTCACAACCGTCCTACCATCTCC	62.8
	2. TCACTGCTGCTTTTTCCACTTCC	63.5
PERL0160919	1. TCTCACCACGTCACGCTTTA	57.3
	2. GCAAAATTCATGTGGGCTTT	57.8
PERL0074777	1. GAACATGCCCTTCTGATGGAGT	60.5
	2. ACTTGAGGTCCTTGTGGCTG	57.3
Ossowski_174629	1. CGCATTGAAGAAAATTGGTAA	56.1
	2. TCGTTGCTTTGACTTCATCG	57
PERL0272288	1. CTCGTCAGCGTCTCTCTCCT	56.7
	2. CGATTGCAGCATCCAGTCTA	57
PERL0376281	1. CCAAATGATCCACCCACTCT	56.8
	2. CCTCCTCCCTGTTGATGTTC	56.5
PERL0349304	1. CAAATGACAACGTACTAGCATAGTC	56.3
	2. TCCTGGCTGATCCTTTTCAT	57
Ossowski_289737	1. AAAGGTCCCATCTGCTTGTG	57.4
	2. TCGGTCTAGTGGGATTACGG	57.5
PERL0468003	1. GGCTTTTTCCGTTGTTTCAA	58.1
	2. TTGTCCCGTTGAGTGTGTGT	56.3
PERL0631952	1. AAGAAAACCAAAGCGGTCAA	57.7
	2. AAGAACCCCTCTACCATTTCG	57.8
PERL0445795	1. GACAGAGGGTTCTTGATAGTT	50.1
	2. TGAATGGTTAGATGGGAAT	49.3
PERL0612640	1. CTCCTGCAACAGTTCTTGTCC	57.2
	2. GCCTATTGATACCACAGAATGAGA	58.2
Ossowski_664016	1. GGTAATGCTCTTTGTGGCTGA	57.9
	2. CCCATTCACTCTCTTCGGCT	59.1
FCA312	1. ATCTCAAACCATCATTATCCAG	53.7
	2. TCAAGAAAGACAATTCAGCTAC	52
PERL0671404	1. ATTGGATGATACGGTGAAAC	52.1
	2. AGCGAGAGGGCACTGTGATA	58
Ossowski_800075	1. TCACAGTCACCAGCCAGAAG	56.3
	2. AGTCCCGAGGAGGATGCT	56.3
PERL0895287	1. TTCGATTTGCAGTGGTTGTG	57.6
	2. TTGAGGAGGAAATGGAAACG	57.6
KLPNHC	1. CGTTGCTCGTGGATTTTGTAA	59
	2. CTTGTATAAGTTCTTGCCTGTGA	54.8
Ossowski_877533	1. GTCCATATTATCGCATCAAAG	52.9
	2. CTGGGAGTGGGAACTACAA	54.9
Ossowski_1057035	1. ATAATCTCTCTCCCGCCTCA	56.4
	2. CGTATGACCAGATTCAAAGCA	56.3

2.1.3. Statistical analysis

Statistical tests for the data were carried out using Minitab 15 software (Minitab Inc, USA). Comparisons for normally distributed data with equal variances were performed using Student's *t*-test or one way (ANOVA) followed by Tukey's multiple comparisons (family error rate = 5). Where data did not follow a normal distribution the analysis was done using the Mann-Whitney *U*-test with the P-value adjusted for ties.

2.1.4. Image capture and processing

Images of the whole plant, inflorescences, and chromosomes were obtained using a Coolpix 4500 digital camera (Nikon). Flowers, siliques, and mature seeds were photographed under a SMZ1500 dissecting microscope (Nikon) using a Digital Sight DS-U1 colour camera (Nikon). Captured images were processed using the Adobe Photoshop Elements Software.

2.1.5. QTL mapping and statistical analysis

QTL analysis was carried out using composite interval mapping (CIM) implemented by the software computer program QTL Cartographer (Basten *et al*., 1994, 2001). Log-likelihood (LOD) thresholds were used to determine the significance of the QTLs. The analyses were performed on the means and principal component scores of 70 tetraploid RILs as pollen parent crossed with Col-0 as seed parent. The CIM analysis was done by P. Kover (University of Bath, UK).

2.2. Methods

2.2.1. Growth conditions for *A. thaliana*

Seeds were stratified in 0.15% agarose at 4°C for 3-5 days. Seeds were sown onto Levingtons F2 compost pre-treated with 0.2gm/L insecticidal intercept solution (Intercept 70 WG - Scotts). Sown seeds were covered with propagator lids for about 10 days after germination. Plants were grown in a glass house with a day length of 16 hours at 24°C (day) and 18°C (night) or in a Sanyo controlled environment room with a day length of 16 hours, 70% relative humidity, 22 °C (day) and 18 °C (night).

2.2.2. Cross pollination

For reciprocal crosses between individual *Arabidopsis* plants, the seed parents were emasculated one day prior to anthesis and pollinated two to three days after emasculation. Pollination was done when the stigmatic papillae were fully extended. Mature pollen from the pollen parent was gently dabbed onto the mature stigmas. The process of emasculation was done carefully by selecting only 3-5 buds per branch, and removing either a few or all the remaining flower buds around the emasculated buds to avoid self- and cross-pollination. Both emasculation and pollination were carried out under a Leica MZ6 dissecting microscope.

2.2.3. Cytological technique for counting mitotic chromosomes

Young *Arabidopsis thaliana* flower buds were selected for chromosome squashs.

2.2.3.1. Fixation

Young auxiliary inflorescences were collected from relevant plants, and the buds were immersed in. freshly made Carnoy solution (3 parts absolute alcohol: 1 part glacial acetic acid). The material was stored in the fixative at 4°C. At least 30 minutes fixation was allowed before hydrolysis.

2.2.3.2. Processing

Following the method of Bailey and Stace (1992), the plant material was removed from fixative and hydrolysed for 10 minutes in 5N HCl. After hydrolysis the inflorescences were removed from HCl and placed in freshly prepared 70% ethanol. The inflorescences were then dissected using fine pair of needles in a drop of 45% glacial acetic acid under a dissecting microscope. The material was then placed in a drop of 1% aceto- orcine (1gm orcein: 55 ml glacial acetic acid: 45 ml distilled H_2O), covered with a coverslip and heated from beneath for 4-5 seconds for staining. The slide was placed in a fold of filter paper, placed on a flat surface and the squash was made by applying pressure using the thumb. The slide was examined and chromosome counts done using a Phase Contrast Microscope (Olympus, BH-2, Japan).

2.2.4. Phenotypic characterisation

Phenotypic characterisation of *moa-1* mutant plants was done by growing the mutant plant and its wild type control L*er,* under the same growth conditions and scoring them for variation in size and shape of the leaf and stem, flower size and petal number, silique length, and seed size and shape.

2.2.5. Seed weight

After approximately 8 weeks from sowing, mature seeds were harvested from dry siliques that were naturally desiccated. Occasionally, seeds were stored in a sealed container with silica gel to ensure complete dessication. Mean seed weight was obtained by weighing all the seeds from 3-6 individual siliques from 3 different plants. Seed weights were measured using a Mettler UMT2 microbalance (Mettler-Toledo, Leicester, UK).

2.2.6. Categorising the seeds

Once the mature seeds were collected from different ecotype crosses, they were separated and categorised into two groups: plump and shrivelled. The process was done by eye under light microscope.

2.2.7. Seed germination

Two filter disks (Grade1, size 8.5cm, 90mm, Whatmann) were placed into a Petri dish. A glass microfiber filter paper (GF/A, 70mm, Whatmann, UK) was place on top and 5ml of sterile milli-Q water was added to the Petri dish. Seeds were placed evenly spaced into the Petri dish and sealed with parafilm (M, UK). The petri dishes with the seeds were placed in a Sanyo control environment room with day length of 16 hours, 70% relative humidity, 22 °C (day) and 18 °C (night) for approximately two weeks. After 2 weeks the seeds were scored for germination (i.e. when the radicle and cotyledonary leaves are formed), and representative sample photographs taken.

2.2.8. Making tetraploids from diploids

Tetraploids of *Arabidopsis thaliana* ecotypes, CSS lines and RILs were generated by treating two-week old seedlings with 6 µl of either 0.125% or 0.25% colchicine (Sigma). Treated plants were grown to maturity scored for phenotypic alteration to flowers, inflorescence and seed sizes to validate the success of colchicine treatment. Seed was harvested from plants showing the appropriate morphology (large flowers) and increase in seed size (area and weight) compared to the corresponding wild type. A small number of plants were grown from each putative tetraploid line and subjected to karyotype analysis (method section 2.2.3) to confirm the appropriate ploidy level.

2.2.9. Seed clearing and differential contrast (DIC) microscopy

Seeds after 3, 5, 6, 7 DAP were dissected from siliques and mounted in clearing solution (chloral hydrate/ water/ glycerol, 8:3:1). They were then left for 1-6 hours to clear depending on the number of days after pollination. Cleared seeds were examined under a Nikon 90i Eclipse microscope (Tokyo, Japan), using differential interference contrast optics. The slides were imaged and photographed with a DS-U1 colour camera (Nikon).

2.2.10. Feulgen staining and confocal microscopy

Feulgen staining of seeds was carried out according to the method of Braselton *et al.*, (1996). *Arabidopsis* siliques (3, 5, 7, or 9 DAP) were placed in freshly prepared Carnoy's fixative solution (3 ethanol: 1 acetic acid) and stored overnight at 4 °C. Siliques were removed from the fixative solution and transferred to 70% ethanol where they could

be kept at 4 °C for several weeks before use. A 3 day protocol for staining and slide preparation was followed.

Day 1: siliques were washed 3 times in sterile milli-Q water for 15 mins to remove any remaining ethanol before hydrolysis in 5N HCl for 1 hour, this was followed by three washes in sterile milli-Q water for 10 mins each. To avoid any tissue damage, after the last wash Schiff's reagent was pipetted in the same tubes and they were incubated at room temperature in the dark for 2 hours. Each silique, now stained pink, was individually washed in cold tap water 3 times for 10 mins. The samples were then taken through an ethanol series: 1 wash in 70% and 95%, two washes in 100%, each for 10 mins. Samples were then incubated overnight in fresh 100% ethanol at 4 °C.

Day 2: The 100% ethanol was changed every hour till no pink colour leached from the siliques. The siliques could be stored for several weeks at -20 °C at this point). Following this, the ethanol was replaced with 1 ethanol: 1 LR White Resin (Agar Scientific, UK) for 1 hour with occasional mixing by gently inverting the tube. The ethanol/LR White resin mix was replaced by pure resin for 1 hour, again with occasional gentle mixing. A final change with fresh pure resin followed and the samples were stored overnight at 4 °C.

Day 3: A drop of resin containing LR White Accelerator (Agar Scientific) was placed in the centre of a microscope slide and two coverslips were pushed partway into it from either side. The stained silique was placed on the slide within the resin droplet and opened with two shallow longitudinal cuts on either side of the septum. Seeds were gently dissected from the pod into the resin using fine needles and the pod wall removed using fine forceps. A third coverslip was then placed over the seeds with its edges supported by the other two coverslips. Once the resin was polymerised, the slides were kept aside for microscopy at a later time.

The Feulgen stained seeds were imaged with an argon ion laser, 488-nm excitation and 515/530-nm emission, using a Nikon C1 confocal microscope system with a 90i Eclipse microscope and EZC1software (Nikon UK). Images were saved as TIFFs and processed with Adobe Photoshop.

2.2.11. Plant DNA extraction

Plant DNA extraction for genotyping was done according to a modified protocol of Edwards *et al.*, (1991). The method was carried out by placing two or three small, young leaves in 1.5 ml tube on ice with a toothpick of glass beads (Sigma) and 450 µl extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl and 25 mM EDTA). A bench drill with a sterile plastic pestle was used to grind the leaf material to fine slurry, after which the debris was pelleted by centrifugation at maximum speed (14,000 rpm) in bench-top centrifuge. The supernatant was decanted to a new tube and subjected to a second centrifugation step and the clear supernatant was carefully pipette out in a fresh eppendorf tube. An equal volume of 100% isopropanol was added to the supernatant, followed by incubation at room temperature for 2-5 minutes. DNA pellets were obtained by centrifugation at speed (14,000 rpm) for 10 minutes. The pellet was washed three times with 70% ethanol, and left to dry in laminar flow hood for 5 minutes or until all traces of liquid had evaporated. The pellet was dissolved in 80µl sterile milli-Q H_2O and the sample stored at 4 °C for use with 2-3 months.

2.2.12. DNA amplification for genotyping analysis

Each 25 µl reaction contained 2 µl of extracted DNA solution, 17.75 µl sterile milli-Q H_2O, 2.5 µl of 10x PCR buffer (100 mM Tris-HCl pH8.3, 500 mM KCl, 15 mM $MgCl_2$), 0.5 µl dNTPs mix (10 mM),1µl of each forward and reverse primers (10 µM), and 0.25 µl *Taq* DNA polymerase (5 U/ µl). All reactions were carried out on a PTC- 200 Peltier Thermal Cycler (MJ Research, USA). The amplification programme consisted of: Initial denaturation at 94°C for 4.0 min, followed by 32 cycles of denaturation at 94 °C for 30 seconds, annealing at X°C (where X depends on the primer pairs used) for 30 seconds and extension at 72 °C for ~ 1 min (depending on the fragment length, 1 min/kb)., A final extension for 10 min at 72 °C was done to finish off the PCR products. Details of PCR primer sequences used for genotyping are shown in (Table 2.4). The PCR product was cleaved with appropriate enzymes to ascertain the genotype.

Table 2.4: Primer sequences, position of markers, and restriction enzymes used for genotyping analysis

Marker name	Primers, 1=forward, 2= reverse	Amplicon length	Tm (°C)	Position	Ext. Time(sec)	Restriction Enzyme
BKN000001028	1. TTCACAACCGTCCTACCATCTCC 2. TCACTGCTGCTTTTTCCACTTCC	622bp	62 ºC	Chromosome 1	37	StyI
PERL0160919	1. TCTCACCACGTCACGCTTTA 2. GCAAAATTCATGTGGGCTTT	880bp	55 ºC	Chromosome 1	52	HpaII
PERL0074777	1. GAACATGCCCTTCTGATGGAGT 2. ACTTGAGGTCCTTGTGGCTG	875 bp	60 ºC	Chromosome 1	53	KpnI
Ossowski_174629	1. CGCATTGAAGAAAATTGGTAA 2. TCGTTGCTTTGACTTCATCG	906 bp	57 ºC	Chromosome 1	55	HindIII
PERL0272288	1. CTCGTCAGCGTCTCTCTCCT 2. CGATTGCAGCATCCAGTCTA	971bp	55 ºC	Chromosome 2	56	HincII
PERL0376281	1. CCAAATGATCCACCCACTCT 2. CCTCCTCCCTGTTGATGTTC	986bp	56 ºC	Chromosome 2	59	ApaII
PERL0349304	1. CAAATGACAACGTACTAGCATAGTC 2. TCCTGGCTGATCCTTTTCAT	887 bp	56 ºC	Chromosome 2	54	HinfI
Ossowski_289737	1. AAAGGTCCCATCTGCTTGTG 2. TCGGTCTAGTGGGATTACGG	915 bp	57 ºC	Chromosome 2	55	DdeI
PERL0468003	1. GGCTTTTTCCGTTGTTTCAA 2. TTGTCCCGTTGAGTGTGTGT	926bp	52 ºC	Chromosome 3	56	SspI
PERL0631952	1. AAGAAAACCAAAGCGGTCAA 2. AAGAACCCCTCTACCATTTCG	852bp	55 ºC	Chromosome 3	56	ClaI
PERL0445795	1. GACAGAGGGTTCTTGATAGTT 2. TGAATGGTTAGATGGGAAT	1172 bp	50 ºC	Chromosome 3	70	Hpy188I
PERL0612640	1. CTCCTGCAACAGTTCTTGTCC 2. GCCTATTGATACCACAGAATGAGA	930 bp	57 ºC	Chromosome 3	56	AvaII
Ossowski_664016	1. GGTAATGCTCTTTGTGGCTGA 2. CCCATTCACTCTCTTCGGCT	912 bp	58 ºC	Chromosome 4	55	NdeI
FCA312	1. ATCTCAAACCATCATTATCCAG 2. TCAAGAAAGACAATTCAGCTAC	1117bp	52 ºC	Chromosome 4	67	EcoRI
PERL0671404	1. ATTGGATGATACGGTGAAAC 2. AGCGAGAGGGCACTGTGATA	1072 bp	56 ºC	Chromosome 4	65	DraI
Ossowski_800075	1. TCACAGTCACCAGCCAGAAG 2. AGTCCCGAGGAGGATGCT	861 bp	56 ºC	Chromosome 4	52	EcoRV
PERL0895287	1. TTCGATTTGCAGTGGTTGTG 2. TTGAGGAGGAAATGGAAACG	934bp	52 ºC	Chromosome 5	56	HincII
KLPNHC	1. CGTTGCTCGTGGATTTTGTAA 2. CTTGTATAAGTTCTTGCCTGTGA	910bp	54 ºC	Chromosome 5	55	EcoRI
Ossowski_877533	1. GTCCATATTATCGCATCAAAG 2. CTGGGAGTGGGAACTACAA	1091 bp	54 ºC	Chromosome 5	66	DraI
Ossowski_1057035	1. ATAATCTCTCTCCCGCCTCA 2. CGTATGACCAGATTCAAAGCA	918 bp	57 ºC	Chromosome 5	55	HpaII

2.2.13. Agarose gel electrophoresis

Agarose gels were made by dissolving electrophoresis grade agarose (from either Invitrogen or Fisher, UK) in 1x TAE buffer (40 mM Tris, 1 mM EDTA, glacial acetic acid (1.14%), pH 7.6), and adding ethidium bromide to a final concentration of 0.2 µg/ml before setting. Gels were left to set for 30-60 min, and then submerged in 1x TAE buffer containing ethedium bromide in gel Sub-Cell tank (Bio-Rad). Prior to loading of samples, an appropriate volume of 6x loading dye was added to each. Either a 1 kb or 100bp DNA ladder (New England BioLabs) was loaded on each gel as a molecular weight marker. Electrophoresis was normally carried out using a Powerpac 300 power supply (Bio-Rad) at ~ 100 V. The DNA bands were visualised on a UV transilluminator (UVP, USA).

2.2.14. Construction of a genetic linkage map

Construction of linkage map requires:

a) Development of appropriate mapping population and deciding the sample size.

b) Selection of suitable markers for genotyping the mapping population.

c) Screening parents for marker polymorphisms and then genotype the mapping population (parents and the progenies).

d) Linkage analysis (Calculate the recombination frequencies between markers, establish linkage groups, estimate a map distances, and determine a map order) using specific statistical programmes.

2.2.15. Mapping population

The mapping population was developed to study the killing effect caused by Col-0 ecotype. A cross was made between Tsu-1 (maternal modifier parent) and Col-0 *A. thaliana* ecotypes. The F_1 was left to self fertilise to produce F_2 population, both populations were screened for plump and shrivelled seeds by crossing them with Col 4x pollen parent. The F_2 plants were backcrossed (BC) to the recurrent parent Tsu-1 for the highly submissive, and to Col-0 for the highly resistant plants. The new generated $BC1F_1$ plants were categorised to either resistant or submissive by pollination with Col 4x. The highly resistant and submissive $BC1F_1$ plants were then harvested individually and left to

self fertilise to produce $BC1F_2$. These plants were crossed with Col 4x (pollen parent) to select both highly resistant and highly submissive plants. $BC1F_2$ highly resistant and highly submissive plants were again backcrossed to both Col-0 and Tsu-1 as described before to generate $BC2F_2$ plants. The process of backcrossing, self fertilisation, Col 4x pollination, and selecting both highly resistant and highly submissive plants was used to produce $BC3F_3$ progenies which were then used for genetic characterisation, see more details in chapter 5 (Figure 5.9).

Chapter 3

3. The genetics and characterisation of *A. thaliana MOA* gene

3.1. Introduction

3.1.1. Cell cycle control

The cell reproduces and duplicates itself through a series of events called the cell cycle. It is the basis of reproduction and sustained growth of all living organisms. The cell cycle requires two major events, S-phase and mitosis, both of them ensure that the newly formed cells receive a full complement of chromosomes. Defects in these events result in the formation of daughter cells that will not receive the full set of genes required for all cellular activities. These cells either die or sustain genetic damage and so can no longer function correctly (Nurse, 1990).

3.1.2. Mitosis and its role in cell division

Mitosis has been studied since the early 1880s. It is a fundamental process for life in which all the cell contents, including its chromosomes, duplicates and splits to form two identical daughter cells. The transmission of the genetic material from the mother cell to daughter cells must be executed in a coordinated and precise manner.

Mitosis is a complex process consisting of numerous inter-related steps, including chromosome condensation, spindle formation, centromere attachment to the spindle, and separation of sister chromatides and chromosome movement toward the poles, followed by elongation of the spindle and decondensation of the chromosome (Samejima *et al.,* 1993). Mitosis is controlled by a number of genes, however, the most common genes controlling mitotic division are cyclin-dependent kinase (CDK) complexes and their regulatory subunits (cyclins) (Jacobs, 1995; Dewitte and Murray, 2003). Errors in regulating mitosis lead to a disorganised mitosis with a change in the genetic information in the daughter cells. Compton (2005) stated that accurate chromosome segregation is important for the mitotic phase of the cell cycle and failure in this process leads to aneuploidy, which results in the initiation and progression of cancer.

The mitosis mechanism is universally conserved in eukaryotes, but specific solutions to achieve this process have been adapted by different organisms during evolution (Criqui and Genschik, 2002). Although cytological studies of plant cells have contributed to our understanding of mitotic chromatin dynamics, many of the molecular mechanisms that control mitosis have been identified in animal and yeast cells (Criqui and Genschik, 2002). Many researchers such as Nurse (1990) and Yanagida (1989) isolated a number of mitotic mutants of the fission yeast *Schizosaccharomyces pombe* which are useful in understanding the control of mitosis. In fission yeast the regulation of the mitotic onset involves at least four gene functions acting in a network together (Nurse, 1990).

Cyclin-dependent kinases (CDKs) that are activated by mitotic cyclins are the key players in mitotic division (Pines, 1999). Nevertheless, several regulatory kinases and phosphatases, as well as other regulatory proteins and structural components, are involved in the regulation of mitosis (Pines, 1999). Genes that are involved in the control of cell division and growth are variable. Spellman *et al*., (1998) found that ~800 genes are cell cycle regulated, which constitutes more than 10% of all protein coding genes in the genome.

3.1.3. Aneuploidy

Aneuploidy is the condition of having less than or more than the normal diploid number of chromosomes, and is the most frequently observed type of cytogenetic abnormality. Aneuploidy has played a central role in plant genetics. It is a very specialized subject of both theoretical and practical importance. It has been found that doubling of the entire chromosome complement of a plant usually has little effect on fertility, while addition of a single chromosome may result in very drastic changes to the phenotype and could lead to sterility (Henry *et al*., 2005). Furthermore, plants are the best organisms for studying the effects of aneuploidy on gene stability and expression (Khush, 1973; Papp *et al*., 1996) because many species tolerate the presence of extra chromosomes especially polyploidy.

3.1.3.1. Aneuploidy theory

Aneuploidy is any deviation from euploidy in which the cell may have more than or less than the normal diploid number of chromosomes during the fusion of gametes in the meiotic division. It is the most frequently observed type of cytogenetic abnormality. The two most commonly observed forms of aneuploidy are monosomy (2n-1) which is the lack of one of a pair of chromosomes and trisomy (2n+1) that refers to an individual having additional chromosome to the normal diploid number (Hartwell *et al.* , 2004). Lutz (1909) was the first to report aneuploid chromosomes in the hybrid between *Oenothera lata* and *Oenothera gigas.*

Tackholm (1922) was the first to introduce the term aneuploidy following her research on *Rosa* genus (Koul and Dhar, 1998). Aneuploids have played a major role in plant genetics since it was reported a considerable time ago in *Avena* (Khush, 1973), *Oenothera* (Lutz, 1909) and *Datura* (Blakeslee, 1921) species. Aneuploidy also received attention after the classic studies of *Datura* trisomics by Blakeslee and co-workers (Blakeslee, 1921; Blakeslee *et al*., 1920). Trisomics are useful for assigning genes to specific chromosomes and for studying the effects of extra chromosome on the characters of phenotype (Khush, 1973; Papp *et al*., 1996).

Khush (1973) also found that addition of a single chromosome can result in very drastic changes to the phenotype that results in sterility. In human genetics, chromosome abnormality (Hassold *et al*., 1993) and solid tumor cells (Nowell, 1976) are most commonly aneuploids. The deleterious nature of chromosomal imbalance is also highlighted by the fact that cancerous cells are aneuploidy and that aneuploidy may be a cause of cancer (Matzke *at al*., 2003; Pihan and Doxsey, 2003). Furthermore, the structure and number of each chromosome in many cancer cells can be highly variable (aneuploid), (Storchova and Pellman, 2004). Henry *et al.*, (2007) stated that most aneuploids in humans are embryo-lethal, and several developmental effects are associated with the few viable ones. Henry *et al*., (2006) showed that aneuploidy is deleterious and introduces gene dosage imbalance on a chromosomal scale that is proposed to cause change to phenotypes. In addition, dosage imbalance caused by aneuploidy is generally less sensitive in plants than animals (Henry *et al.*, 2007).

3.1.3.2. Factors causing aneuploidy

Although there has been much attention paid to aneuploidy in plants, there has been little progress in understanding the mechanisms of induction and the environmental changes that cause this process. Approximately 60 plant species were reported by Sharma (1990) as producing mitotic or meiotic aneuploid cells after their treatment with various agricultural chemicals, drugs, natural substances, or industrial products. During cellular segregation, such as respiration, doubling of chromosomes, spindle and phragmoplast functions might be stressed by the previous factors.

Moreover, several metabolic causes such as soil nutritional contents, irrigation by polluted water, plants pathogens, the aging of seeds, pesticide application and residues exhibited aneuploidy in about 45 plant species without any apparent cause or source of stress. Furthermore, some environmental factors such as temperature and pH might play a major role in increasing aneuploidy in plants.

3.1.3.3. The relationship between aneuploidy and triploidy

Triploids were used in the early 1930s as a source of trisomics in different plants such as *Datura* (Blakeslee, 1921), tobacco (Clausen and Cameron, 1944), maize (McClintock, 1929), and tomato (Lesley, 1928). The idea that triploids could produce viable aneuploids was confirmed by these studies and most of the produced aneuploids were trisomics. Ramsey and Schemske (1998) stated that triploids are functioning as bridges between diploid and tetraploid populations. Particularly, triploid progeny might be composed of a complex swarm of varied karyotypes having a different number of chromosome copies (Henry *et al*., 2005).

There is a variation for the production of aneuploids and triploids in plants. Cherry tomato of the San Marzano variety produces more aneuploids than triploids (Rick and Baron, 1953; Rick and Notani, 1961). These variations were also greater between species such as poplar (Johnsson, 1945), sugar beet (Levan, 1942), and *Melandrium* (Warmke and Blakeslee, 1940). In addition, Henry *et al*., (2006) found that triploid *A. thaliana* plants generated by crossing diploids with either a natural or induced tetraploid produced a swarm of viable aneuploid progeny following selfing.

3.1.4. Mosaic aneuploidy

Chromosomal mosaicism was originally defined as the presence within the same individual of cells differing with respect to their chromosome complement (Montalenti, 1978). The same phenomenon is also known as intercellular or somatic genomic variation and mosaic aneuploidy. This later term will be adopted here. Mosaic aneuploidy is a relatively frequent occurrence in human genetic disorders, even though it is difficult to detect subtle mutant cell lineages (Yurov *et al*., 2007). They also found that the presence of mosaic chromosome abnormalities occur in children with syndromic autism (a devastating psychiatric disorder in childhood). In addition, Lightfoot *et al*., (2006) showed that chaotic mosaic aneuploidy, which consists of mosaic cells within a developing embryo resulting from random chromosomes allocation to daughter cells, is the most common mosaic aneuploidy found at embryo preimplantation. Moreover, Hanks *et al*., (2006) found that *BUB1B* mutations (*Budding uninhibited by benzimidazoles 1 homolog beta*) are associated with mosaic variegated aneuploidy (MVA) and unlikely to be common in sporadic childhood cancers.

3.1.4.1. The behaviour of *moa-1* (mosaic aneuploidy-1)

The *moa-1* mutant of *A. thaliana* was obtained in a screen of ethyl methane sulphonate (EMS) mutagenised seeds of Landsberg *erecta* (L*er*). A PhD student (Heidi Godwin) in the Scott Lab at the University of Bath used a seed size selection screen to process mutant seed from the LEHLE Seeds (Round Rock, Texas, USA). Sieves of different mesh sizes were used to separate abnormally large seeds from normal sized seeds. One abnormally large seed was used to establish an apparently stable putative mutant line that preliminary karyotype analysis suggested was a mosaic aneuploid. The mutant was therefore termed mosaic aneuploidy-1 (*moa-1*).

Studying the mosaic aneuploidy *moa-1* was undertaken for two reasons. First, there were no reports of mosaic aneuploidy in plants and therefore *moa-1* presented an opportunity to study the phenomenon at the cell and molecular biological levels. Second, previous work in the Scott lab had investigated the impact on *A. thaliana* seed size of varying parental contributions to the endosperm at the level of whole genomes; *moa-1* therefore offered the opportunity to construct and analyse endosperms with more subtle levels of genomic variation.

3.3. Results

3.3.1. Phenotypic traits

In order to study the phenotype characters of wild type and the *moa-1* mutant plant, both plants were grown at the same time and under the same growth conditions to record any differences in their features. *moa-1* plants differed from wild type plants for phenotypic characters including rate of rossette growth, flower size and petal number (Figure 3.1). The most frequent differences were visible in the inflorescence of *moa-1*, which carried larger flowers that sometimes had 4 or more petals compared to the normal 4 petals in wild type plants (Figure 3.1b, e and g).

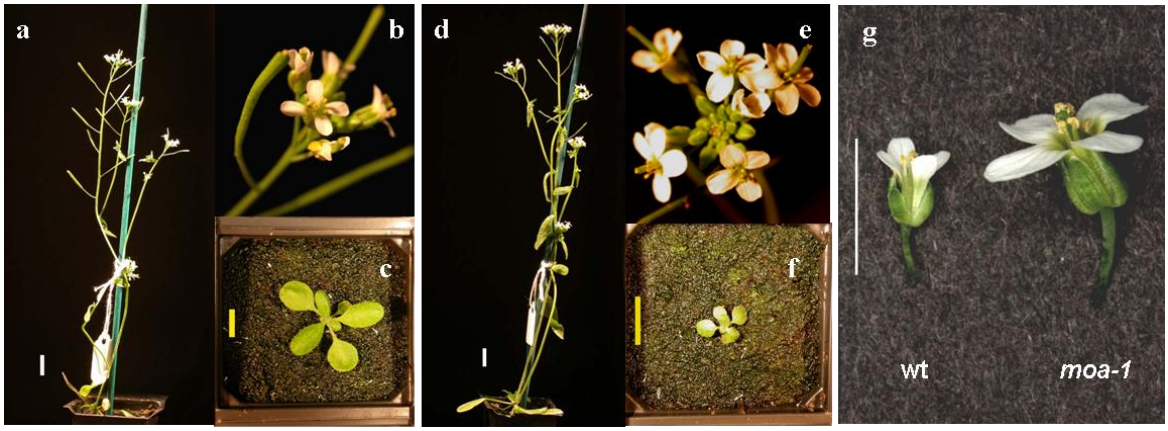

Figure 3.1: Morphology of wt and *moa-1* plants (a) Whole plant of wt after 32 days growth, (b) Inflorescence of wt with normal petal number, (c) 18 day old wt rosette, (d) Whole plant of *moa-1* after 32 days, (e) Inflorescence of *moa-1* with excess petal number, (f) 18 day old *moa-1* rosette, (g) Flowers of both wt and *moa-1*. Scale bar is 1 cm.

As shown in Table 3.1, measurements of the mean number and the mean weight of seeds/silique for both wild type and *moa-1* plants were made. From total 10 siliques, the mean number of seeds/silique in *moa-1* plants was significantly (~ 17 seeds; Figure 3.2a) less than that in wild type plants (~ 51 seeds; Figure 3.2b) (Student t-test: $p<0.001$). In contrast, 20 *moa-1* plants displayed significantly higher mean seed weight (33 µg) compared to that in wild type plants (20 µg) (n=20 seeds, Student t-test: $p<0.001$). Apparently, the reduced number and heavier weight of seeds/silique in *moa-1* compared to wild type demonstrates why the siliques were shorter in *moa-1* than in wild type plants (Figure 3.2c). From the above results, we concluded that the simplest and most reliable

phenotypic differences between *moa-1* and wild type plants were the size of both the flowers and the seed.

Table 3.1: The mean number of seeds and weight/silique in both wild type and *moa-1* plants.

Type	Mean no. of seeds / silique± se (n=silique number)	Mean weight of seeds / silique (µg) ± se (n=seed number)
wt	51.3 ± 0.8 (n=10)	20.1 ± 0.5 (n=20)
moa-1	16.8 ± 1.6 (n=10)	33.3 ± 1.0 (n=20)

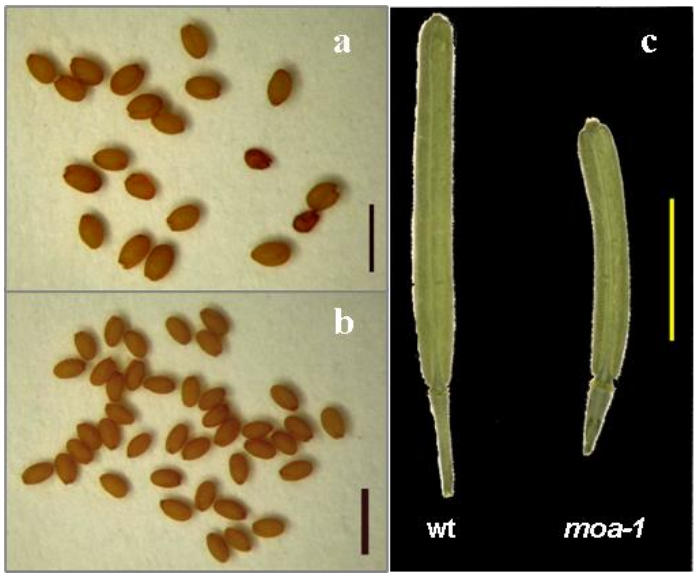

Figure 3.2: Phenotypic traits of wild type and *moa-1*. (a) Mature seed of *moa-1*, (b) Mature seed of wild type, (c) Siliques of both wild type and *moa-1*. Scale bar for a, b is 1mm and 1cm for c.

3.3.2. From genotype to karyotype

In order to study the chromosome number of *moa-1* plants, the cells of *moa-1* plants were karyotyped and the chromosome number compared with that of wild type plants where 2n=10. The experiment was performed on young auxiliary inflorescences from both *moa-1* and L*er* 2x to obtain the apical meristems. These inflorescences were fixed for squash preparation. Ten different samples were taken from ten different plants of *moa-1* and wild type (2 inflorescences from each plant). As shown in Figure 3.4a and Figure 3.5a, the wild type samples contained cells which invariably had 10 chromosomes. In contrast, individual *moa-1* plants contained cells that had chromosome numbers in the range 11-18; (Figure 3.4 b-i and Figure 3.5 b). The same *moa-1* plant showed a variable number of chromosomes in two different inflorescences. The aneuploidy also differed in two different *moa-1* plants. For example, twelve, thirteen, fifteen, and sixteen chromosomes were observed in different cells of one plant (Figure 3.4 b-e), whereas, eleven, fourteen, seventeen, and eighteen chromosomes were observed in a second plant (Figure 3.4 f-i).

The karyotype analysis of *moa-1* plants as demonstrated in Figure 3.5b also showed that ~ 30 % displayed 11-16 and 11-18 chromosome range; whilst, ~ 20 % showed a range between 11-17 chromosomes and ~ 10 % of chromosomal range revealed for both 11-15 and 11-19 chromosomes in *moa-1* plants. These observations appeared to confirm that *moa-1* was a mosaic aneuploid mutant.

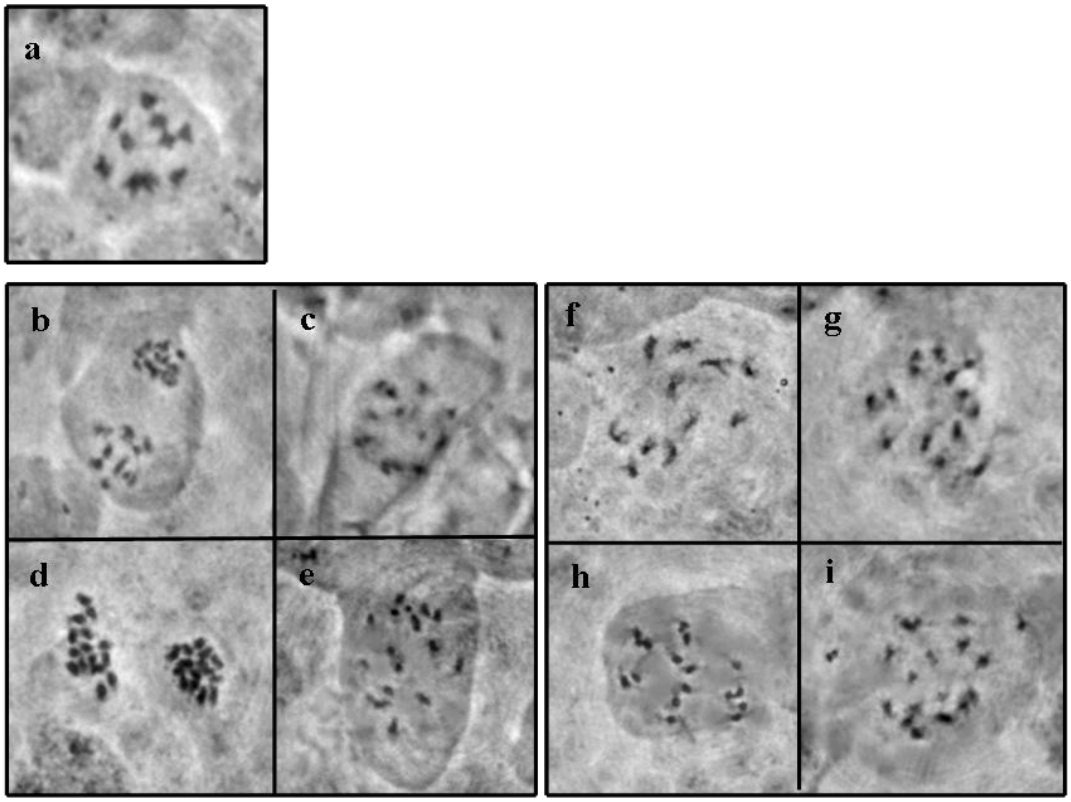

Figure 3.4: Karyotypes of wild type and *moa-1* cells (a) A cell from a wild type plant showing 10 chromosomes (b-e) Cells from *moa-1* plant number 1 showing the range of chromosome numbers; b.12 chromosomes, c.13 chromosomes, d.15 chromosomes, e. 16 chromosomes (f-i) Cells from *moa-1* plant number 2; f.11 chromosomes, g.14 chromosomes, h.17 chromosomes, i.18 chromosomes. (Magnification=100x)

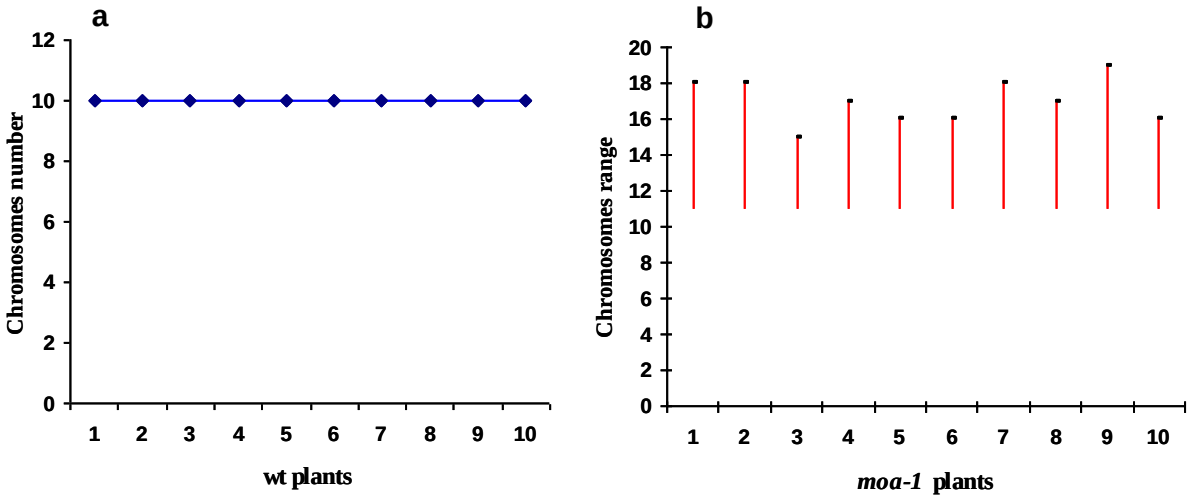

Figure 3.5: Variation for chromosome number or range in 10 different wild type and *moa-1* plants (a) wt **,** (b) *moa-1*.

3.3.3. Genetics of *moa-1*

3.3.3.1. Crosses between *moa-1* and wild type (L*er* 2x) plants

In order to study the genetics of *moa-1*, reciprocal cross pollinations between *moa-1* and L*er* 2x (wt) were performed as shown in Figure 3.6. Fifty F_1 seeds from both crosses were sown. Total of 41 [wt x *moa-1*] and 38 [*moa-1* x wt] plants germinated and were karyotyped during early flowering.

In the first cross between [wt X *moa-1*], the results of the phenotypic characterisation based on flower size of the F_1 progeny showed that 44% had the appearance of wild type plants and 56% had the appearance of *moa-1* plants (Figure 3.7a). In contrast, the reciprocal [*moa-1* X wt] cross yielded 79% wt plants and 21% *moa-1* plants (Figure 3.7b). Although the two crosses yielded different ratios of wild type to *moa-1* plants, the appearance of *moa-1* plants in the F_1 indicated that *moa-1* phenotype is not recessive. Furthermore, the data from the [wt x *moa-1*] cross suggests that *moa-1* was dominant.

The F_1 plants were left for self-fertilisation and the seeds collected to generate F_2 plants. From the phenotypic analysis of the F_2 generation, the results showed that in the cross between [*moa-1* X wt] 15% of the F_2 progeny were wild type and 85% were *moa-1* plants (Figure 3.8a). In contrast, the [wt X *moa-1*] crosses yielded 59% wild type plants and 41% *moa-1* plants (Figure 3.8b). Together with the previous data this suggested that *moa-1* is a dominant character and that the mutant allele may suffer transmission problems especially through the maternal parent (Figure 3.6).

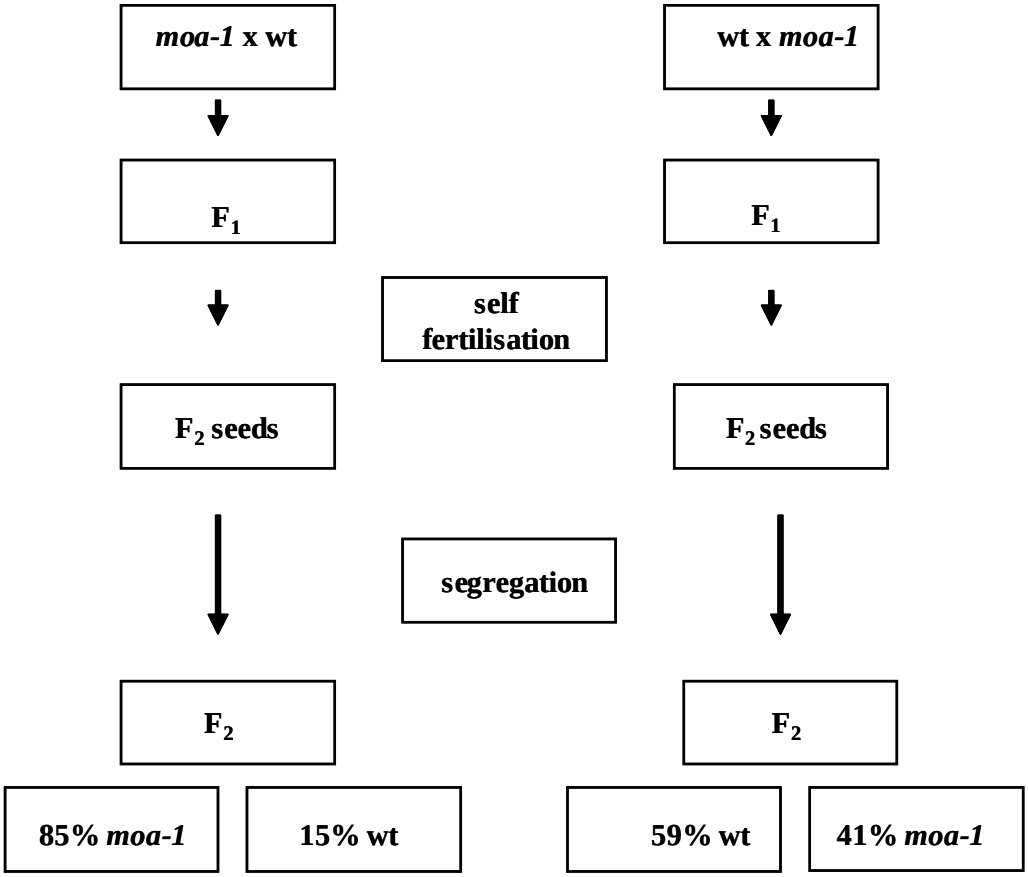

Figure 3.6: Genetics of *moa-1* and the expected segregation in the F_2 generation.

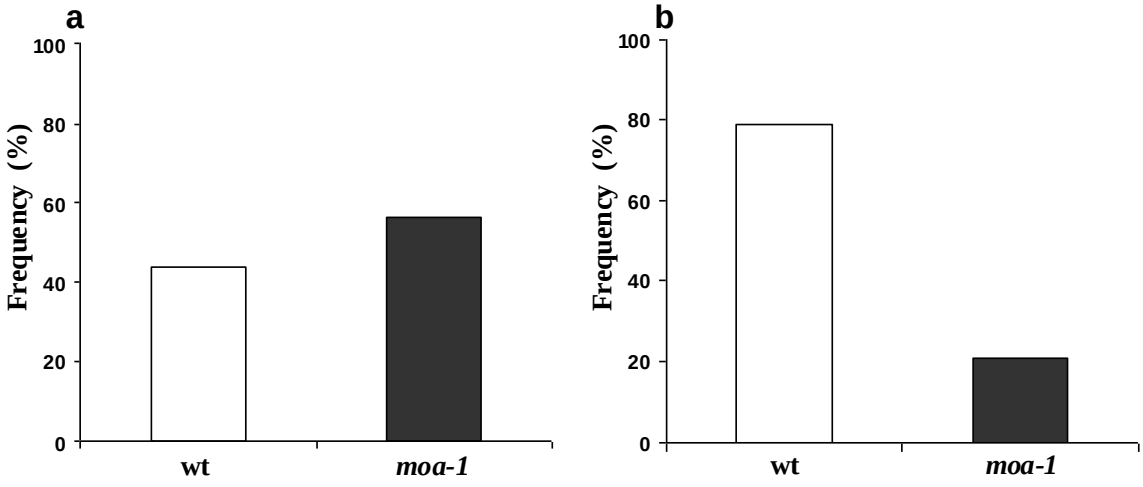

Figure 3.7: Karyotype analysis in the F_1 plants showing the percentage of both wt and *moa-1* looking plants. (a) wt x *moa-1* (b) *moa-1* x wt.

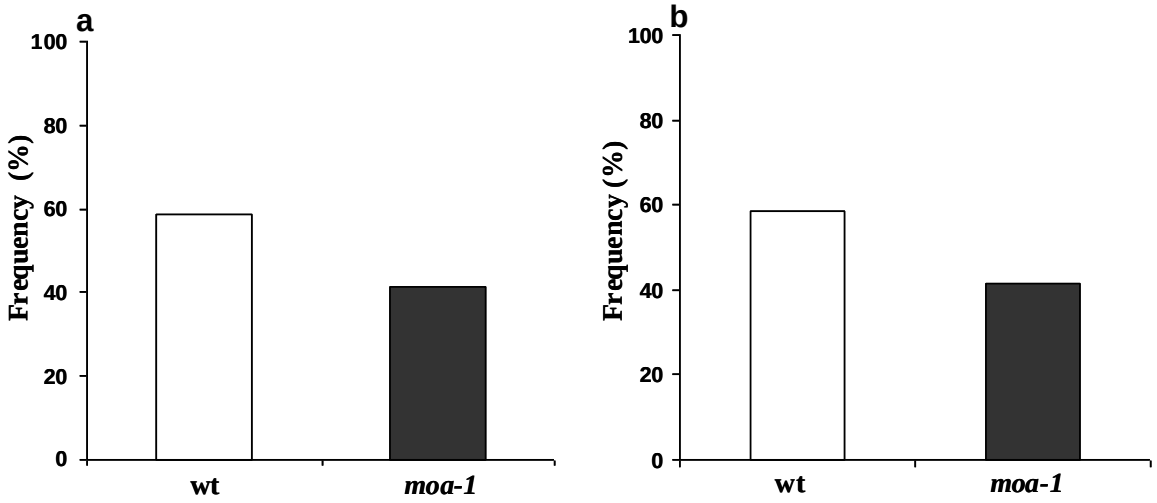

Figure 3.8: Phenotypic analysis of F_2 generation plants showing the frequency of wt and *moa-1* plants. (a) wt x *moa-1* (b) *moa-1* x wt.

3.3.4. Seed viability test

A seed viability test was performed to study the effect of *moa-1* aneuploidy on ability of seeds to germinate. Twenty siliques were collected from four different plants of L*er* 2x, *moa-1*, F_1 plants from reciprocal crosses, and *moa-1* looking plants in the F_2 generation from the reverse crosses.

As shown in Figure 3.9a, *moa-1* produced significantly more shrivelled seeds (~ 9.1 %) than wild type plants (~ 0.3 %). In contrast, the frequency of shrivelled seeds was significantly higher in the F_1 generation in both crosses with less number of plump seeds (~ 31.1 %) in [wt X *moa-1*] cross and (~ 30.6 %) in [*moa-1* X wt] cross; however, this might indicate a transmission problem of *moa-1* in both male through the formation of viable pollen grains and female in developing normal ovules as shown in Figure 3.9b.

In addition, phenotypically *moa-1* plants in F_2 generation had a lower frequency of shrivelled seeds in both crosses with a significant increase in the number of plump seeds. The frequency of shrivelled seeds was ~ 20.2 % in [wt X *moa-1*] cross (Figure 3.10a); while it was ~ 2.8 % in [*moa-1* X wt] cross (Figure 3.10b).

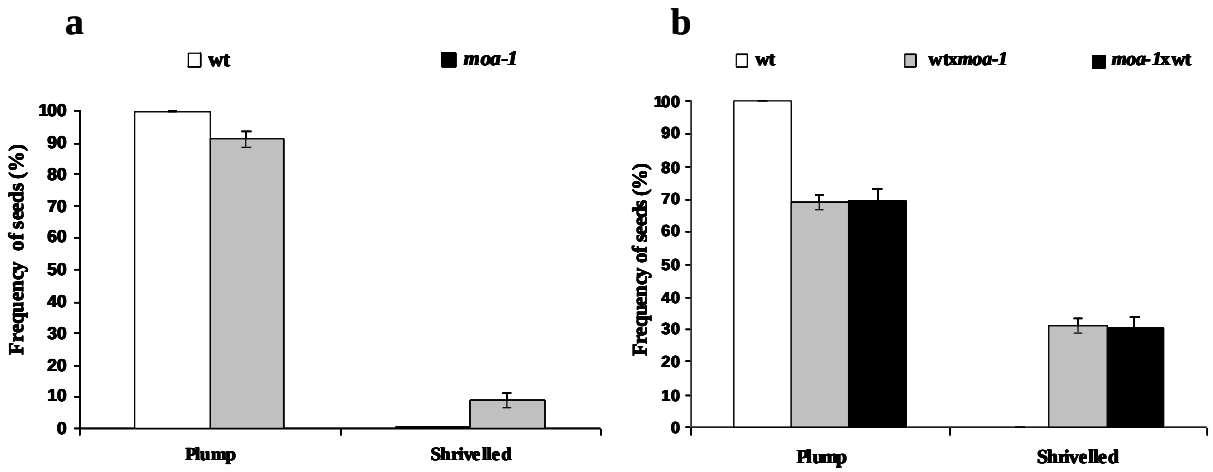

Figure 3.9: Seed development in wt and *moa-1*. The frequency of plump and shrivelled seeds was measured from 20 siliques. (a) Selfed wt and *moa-1*, (b) F_1 plants from reciprocal crosses between wt and *moa-1*.
a,b Significant at $p < 0.001$ (Mann-Whitney test)

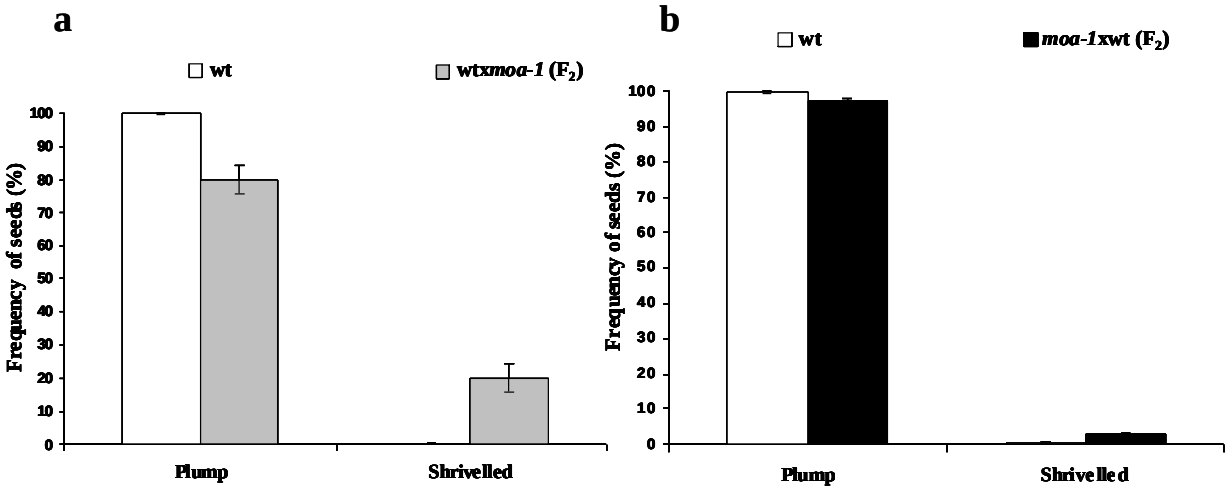

Figure 3.10: Seed developed in F_2 plants from wt and *moa-1* reciprocal crosses. The frequency of plump and shrivelled seeds was measured from 20 siliques. (a) [wt X *moa-1*] cross, (b) [*moa-1* X wt] cross
a Significant at $p < 0.001$ (Mann-Whitney test)
b Significant at $p < 0.001$ (Student t-test)

3.3.5. Endosperm development and genomic imbalance

It was clear from the previous data that *moa-1* affects the viability of seeds which indicated that a change might have occurred in embryo and/or endosperm development. Our preliminary studies of seed development from reciprocal crosses between wild type and *moa-1* showed variation in their size and shape (3.3.1).

Our results from seed development at 5 DAP in the reciprocal crosses between wild type and *moa-1* plants showed that most of the developed seeds in the cross between [wt X *moa-1*] increased in size and the endosperm was overproliferated compared to the wild type seed (Figure 3.11 b-e). In contrast, seeds developed from the crosses between [*moa-1* X wt] showed poor growth of the endosperm and were relatively small compared to wild type seed (Figure 3.11 f-i). Strikingly, a large number of seeds in the [*moa-1* X wt] cross lacked an embryo (Figure 3.11 f and i). According to Scott *et al*., (1998a) an excess of maternal chromosomes in the seed results in poor growth of endosperm and formation of small seeds (e.g. [4x X 2x] crosses) that is similar to [*moa-1* X wt] cross (Figure 3.12a). Whilst, paternal chromosomes excess results in endosperm overgrowth and an increased seed size (e.g. [2x X 4x] crosses) similarly to that observed in [wt X *moa-1*] cross as demonstrated in Figure 3.12b. This confirms the suggestion that *moa-1* have extra chromosomes compared to that in wild type plants.

Since *moa-1* plants have somatic cells with greater than the diploid chromosome number, meiosis and gametogenesis could produce gametes with similarly increased chromosome content. If true, in reciprocal crosses of *moa-1* with wild type plants would result in the same kind of parent-of-origin effects on seed development as observed in reciprocal crosses between 2x and 4x plants. Thus, crosses between wild type and *moa-1* were predicted to provide information about the relative chromosome number in gametes generated in *moa-1* plants.

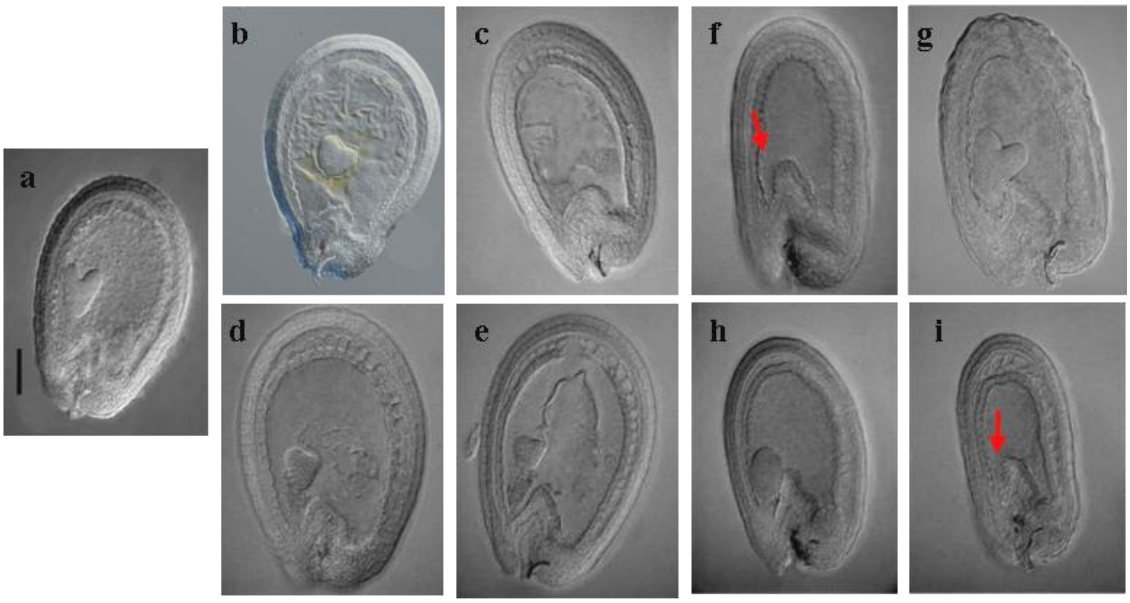

Figure 3.11: Seed development at 5DAP in wt and *moa-1*. (a) wt, (b-e) [wt X *moa-1*], (f-i) [*moa-1* X wt]. Cleared seeds were imaged using differential interference contrast (DIC). Arrow indicates position normally containing the embryo. Scale bar= 100μm.

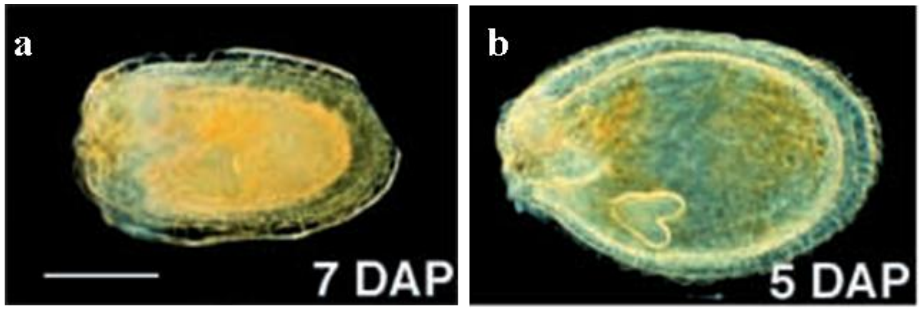

Figure 3.12: Seed development following interploidy crosses (a) [4x X 2x] (maternal excess), (b) [2x X 4x] (paternal excess). Scale bar = 200 μm. (From Scott *et al*., 1998a).

3.3.6. Embryo development in *moa-1* crossed plants

As described above in section (3.3.5), a large proportion of seeds developed following a [*moa-1*X wt] cross lacked an embryo. To better understand the cause of embryoless seed development, the embryo and endosperm was examined at 3, 5, 7, and 9 days of pollination (DAP) in [wt X wt], [wt X *moa-1*], [*moa-1* X wt], and [*moa-1* X *moa-1*] crosses.

At 3 DAP, all the above crosses had seeds with a normal globular embryo i.e. 100% of developed seeds had a globular embryo (Figure 3.13a) (Images of cleared seeds at 3

DAP are not shown). In contrast, at 5DAP (Figure 3. 13b) the frequency of seed with a globular embryo was higher in both the [wt X *moa-1*] and [*moa-1* X *moa-1*] crosses compared to the wild type cross in which almost 100% of the seed had a heart stage embryo. The [*moa-1* X *moa-1*] cross produced seed with the highest frequency (~ 90 %) of globular embryos at this stage. On the other hand, in the [*moa-1* X wt] cross the frequency of globular and heart stage embryo-containing seed was roughly 1:1. Confocal images of cleared seeds for all above mentioned crosses at 5 DAP are shown in Figure 3.15 a, c, e, and g.

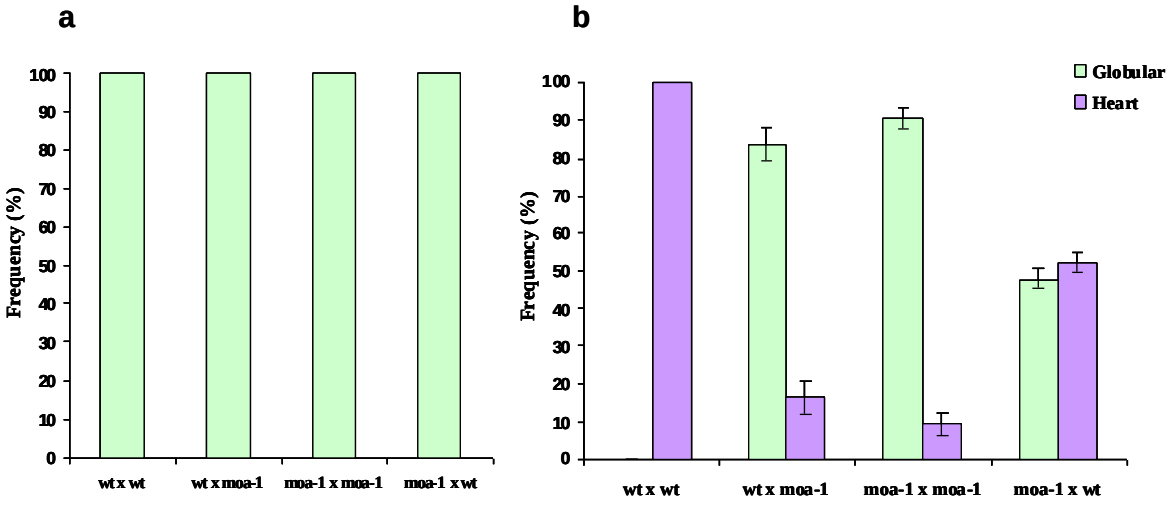

Figure 3.13: Embryo development of wt and *moa-1* crosses. (a) 3 DAP, (b) 5 DAP.

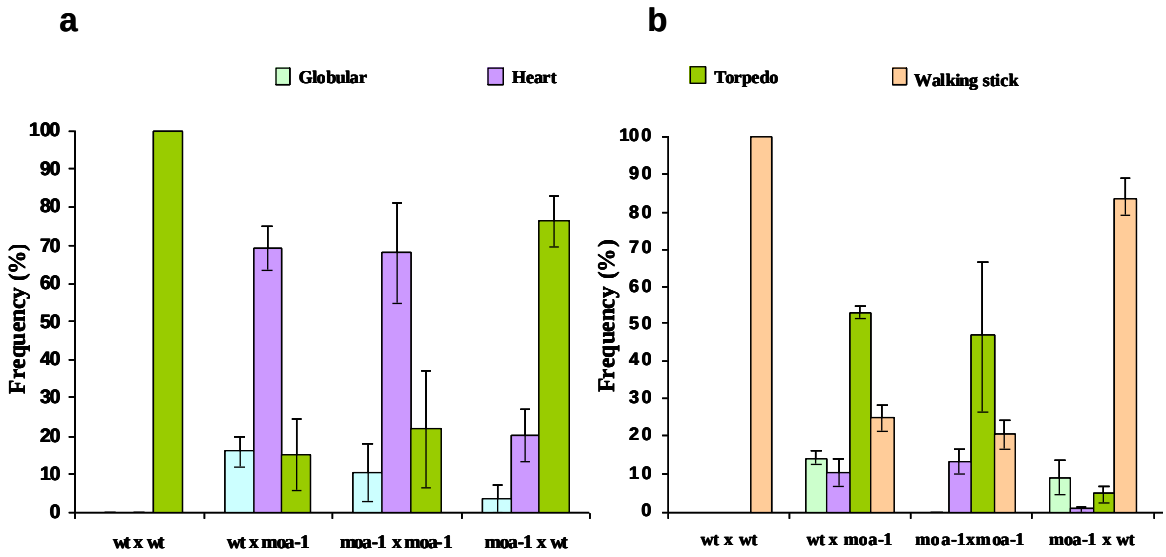

Figure 3.14: Embryo development of wt and *moa-1* crosses. (a) 7 DAP, (b) 9 DAP.

From the data shown in Figure 3.14a it was clear that at 7 DAP the frequency of torpedo stage embryos in the [*moa-1* X wt] cross was substantially higher than both the [wt X *moa-1*] and [*moa-1* X *moa-1*] crosses. The frequency of torpedo embryos in the cross between [*moa*-1 X wt] was (~ 75 %) of total seeds. In contrast, both [wt X *moa-1*] and [*moa-1* X *moa-1*] crosses at 7 DAP produced seed mostly at the heart stage (~ 70 %) compared to the [wt X wt] cross indicating a delay in the formation of torpedo embryos. Seeds with different embryo stages at 7 DAP are shown in Figure 3.15 b,d,f, and h. Seed from [wt X *moa-1*], [*moa-1* X *moa-1*], and [*moa-1* X wt] crosses reached the torpedo stage at 9 DAP (Figure 3.14b). At 9 DAP the majority of seed (~ 85 %) produced by the [*moa-1* X wt] cross had reached the walking stick (WS) stage. However, both [wt X *moa-1*] and [*moa-1* X wt] crosses had torpedo embryos at a frequency (45-50 %) greater than other embryo stages, which indicates a developmental problem from the paternal side caused by *moa-1* mutation; (images of cleared developed seeds at 9 DAP are not shown).

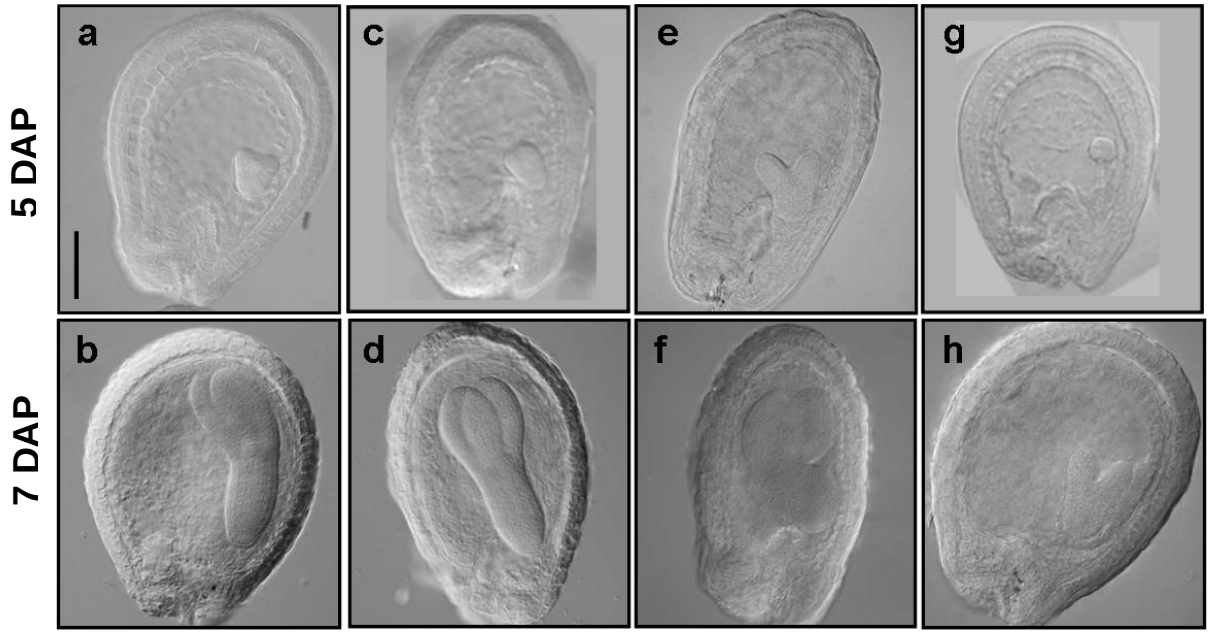

Figure 3.15: Embryo development in seeds at 5 and 7 DAP produced by *moa-1* crosses. (a,b) wild type (c,d) *moa-1* x *moa-1 (*e,f) *moa-1*x wt (g,h) wt X *moa-1*. Cleared seed photos were imaged using differential interference contrast (DIC). [All the seeds are oriented with the embryo on the right and chalazal endosperm to the left]. Scale bar= 100µm.

3.4. Discussion

In the present study a mosaic aneuploidy mutant of the *A. thaliana* (Landsberg *erecta*) was identified from a chemically (EMS) mutagenised seed based on a seed size selection screen. The *moa-1* plants displayed mosaic aneuploidy which is variation in chromosome number in the cells of the same individual. Numerous studies have been performed on aneuploidy, but until the present work there were no descriptions of mosaic aneuploidy in plants.

3.4.1. Characteristics of *moa-1* phenotype

The present study of *moa-1* phenotypic features revealed several easily recognisable differences from the wild type phenotype. Although *moa-1* plants were easily distinguished from wild type plants based on their relatively large overall size, the most reliable phenotypic marker was the large flower size and the extra petals. In addition, the siliques of *moa-1* plants were shorter and contained fewer seeds than those of wild type plants.

3.4.2. *moa-1* is a mosaic aneuploid mutant

To confirm that *moa-1* was a mosaic aneuploid mutant, chromosome karyotyping was the major approach used in this study (3.3.2). As expected, wild type cells of the same or different individuals always contained 10 chromosomes. In contrast, plants that were phenotypically *moa-1* produced karyotypes ranging from 11 to 18 chromosomes. Thus some cells in *moa-1* plants have a larger number of extra chromosomes than is found in the cells of triploid plants in *A. thaliana*. What cannot be determined from the data presented here is the identity of the extra chromosomes: these may be a random set, or biased toward one or a few particular chromosomes.

In humans, Griffiths *et al*., (2000) reported trisomy 16 as the most common aneuploidy; however, they found that fetuses affected with the full version of this chromosome abnormality do not survive. On the other hand, individuals having the mosaic form where trisomy 16 existed in some cells but not all could survive. They concluded that individuals who are mosaic for a chromosomal aneuploidy tend to have a less severe form of the syndrome compared to those with full trisomy. They also found that among many the autosomal trisomies, only mosaic cases can survive.

In contrast to *A. thaliana*, Micale *et al*., (2007) reporting on mosaic variegated aneuploidy (MVA) in humans noted multiple trisomies, but rarely monosomies, suggesting intolerance to large deviations from the diploid complement. Interestingly, MVA caused by *BUBIB* mutations, a condition characterised by constitutional aneuploidies and childhood cancer predisposition, suggest that mosaic aneuploidy can arise due to mitotic spindle dysfunction (Hanks *et al*., 2006; Suijkerbuijk *et al*., 2010).

3.4.3. Allelic dominancy of *moa-1*

To study the genetics of *moa-1* by reciprocal cross pollinations with wild type plants suggested the mutant was dominant or semi-dominant in character. The data from F_2 and F_3 generations largely supported this conclusion.

3.5.3. Endosperm behaviour, embryo development and role of imprinting

Interestingly, data from the crosses between the *moa-1* mutant and wild type plants showed that most of the seeds which were developed from the crosses between [wt x *moa-1*] exhibited a normal endosperm and viable embryos producing normal seed size. In contrast, most of the seed developed in the reciprocal [*moa-1* x wt] was abnormally small with reduced endosperm proliferation and either no embryo or an abnormal embryo. The embryonic autonomy raised a question as to the degree embryo development depends on endosperm size. Data from this work showed that during early endosperm development (3 DAP) embryo growth was similar for both wild type and *moa-1* crosses (Figure 3.14). However, data for later time points (5, 7, and 9 DAP) indicated that paternal *moa-1* delayed the embryo and endosperm growth (Figures 3.15, 3.16, and 3.17).

The viability and final size of seeds in *moa-1* and wild type crosses may reflect the effectiveness of the endosperm in provisioning the embryo during its development. This is supported by Scott *et al*., (1998a) who suggested that the growth of endosperm and embryo are disturbed during interploidy crosses in *A. thaliana* due to numbers of nuclei, timing of cellularisation, or development of certain regions of the endosperm. Moreover, the parental conflict theory predicts that extra doses of paternally expressed loci will result in increased provisioning of the offspring through the endosperm, and this process appears to fail with lethal paternal excess.

The present work did not help understand why the embryo failed to develop in a high proportion of seeds in the crosses between [*moa-1* x wt]. One possibility is that the egg in a proportion of *moa-1* ovules is either absent or abnormal in some way that prevented its fertilisation and subsequent development. Whatever the cause, an autonomous endosperm does successfully develop in embryoless seed. This was most likely the result of fertilisation of the central cell by *moa-1* sperm since autonomous endosperm development in *A. thaliana* occurs only in specific mutant backgrounds e.g. *fis1* (*mea*) (Chaudhury *et al*., 1997) and *fie*/*met1* (Vinkenoog *et al*., 2000).

3.5. Conclusion

The current study confirmed that *moa-1* plant is a mosaic aneuploid and that the *moa-1* phenotype was most likely a dominant trait. There were significant parent specific differences the transmission of the *moa-1* allele. A paternally transmitted *moa-1* allele also had dramatic effects on seed development, including the production of embryoless seed, whereas the maternal allele had little impact on seed development.

This work left many unanswered questions including 1) why *moa-1* caused vegetative and floral over-growth phenotypes, 2) why the frequency of allelic transmission was subject to sex-specific variation, 3) whether the aneuploidy involved specific chromosomes or was random.

Unfortunately, during the course of this study the transmission of the *moa-1* phenotype became highly unpredictable, such that the segregation ratios in potential mapping crosses did not provide confidence that the *moa-1* gene was suitable for map-based cloning. Consequently, all work on *moa-1* was stopped.

4. Genetic variation for triploid block in *A. thaliana*

4.1. Introduction

4.1.1. Triploid block and its discovery

In most flowering plants, the seeds develop abnormally and often abort when parents with different ploidy numbers are crossed (von Wangenheim and Peterson, 2004). Where there is a frequent failure of seed set in the crosses between diploid and tetraploid plants of the same or related species this process is known as the 'triploid block'. Triploid block can result in a high degree of immediate postzygotic reproductive isolation between tetraploids and from their diploid progenitors, since backcrossing to either parent will produce mainly nonviable progeny. This reproductive barrier is caused by malfunction of the endosperm (Köhler *et al*., 2010).

Henry *et al*., (2005) stated that plants can be classified into two categories according to their response to triploidy: in some species, most triploid embryos die because of abnormal endosperm development and the few individuals that develop exhibit highly reduced fertility. Triploid block was first discovered when studying intra-specific diploid and autotetraploid crosses [2x X 4x and 4x X 2x] in different plant species such as maize (Cooper, 1951), brassica (Howard, 1939 and Hakansson, 1956), *Lycopersicon* (Cooper and Brink, 1945), barley (Hakansson, 1953), rye (Hakansson and Ellerstrom, 1950), *Primula* (Woodell and Valentine, 1961), *Galeopsis pubscens* (Hakansson, 1952) and *Poinsettia* (Milbocke and Sink, 1969).

4.1.2. Triploid block and its lethal association in *A. thaliana*

Many flowering plants are polyploids and easily tolerate this condition (Leitch and Bennett, 1997; Scott *et al.,* 1998a). However, in many plant species a 2:1 ratio of maternal to paternal genomes in the endosperm is required for normal seed development (Lin, 1984; Scott *et al*., 1998a; Erilova *et al.*, 2009). Both gene dosage effects and the imprinting of regulatory genes in the endosperm are regarded as the main cause of developmental failure in seeds with abnormal parental contributions (Lin, 1984; Westoby and Haig, 1991; Birchler, 1993; Erilova *et al.,* 2009).

The 2:1 ratio also implies that maternally and paternally derived genomes are not functionally equivalent. Scott *et al*., (1998a) showed that in *A. thaliana* interploidy

crosses, seeds developed from [2x X 4x] or [4x X 2x] were abnormal but viable and showed complementary parent-of-origin seed size phenotypes. The maternal excess cross produced relatively small seeds, whereas large seeds resulted from the paternal excess cross; these phenotypes were correlated to the size of the endosperm. In contrast, seeds generated from the more extreme [2x X 6x] or [6x X 2x] almost all aborted, and were associated with extreme over- or under-proliferation of the endosperm, respectively. Thus, in contrast to many other plant species, *A. thaliana* appears to have tetraploid rather than triploid block.

4.1.3. The relation between triploid block and imprinting

The phenomenon of triploid block is clearly visible in the endosperm, which constitutes a transient tissue of particular sensitivity to ploidy imbalance. The gene dosage balance hypothesis (Lin, 1984; Haig and Westoby, 1991) suggested that parent-of-origin dependent gene expression is responsible for ploidy effects in the endosperm. Thus, the dosage-dependent phenotypes arise as consequence of stoichiometric imbalances in macromolecular complexes, owing to differential parental contributions of dosage-sensitive regulators that are part of these complexes (Birchler, 2001; Veitia, 2003). In *A. thaliana*, mutations in the Polycomb Group (PcG) genes *MEDEA (MEA)* (Grossniklaus *et al*., 1998; Kiyosue *et al*., 1999) and *FERTILIZATION INDEPENDENT SEED2* (*FIS2*) (Chaudhury *et al*., 1997), cause phenotypic abnormalities that are strikingly similar to seed produced in extreme paternal excess [2x X 6x] crosses (Scott *et al*., 1998b; Dilkes *et al*., 2008). Recent studies by Erilova *et al*., (2009) showed that the imprinted expression of *MEDEA* is the underlying cause of the endosperm failure in *A. thaliana* developed seeds that have an increased paternal genome contribution because *MEDEA* is only maternally expressed in the endosperm. Thus, the compartment in the malfunctioning seed that most likely lacks *MEDEA* activity is the endosperm, and as a result, development of the embryo aborts. Hence, the parent-of-origin effects of *medea* mutations on embryo development are likely to be indirect and result from defects in endosperm function (Kinoshita *et al*., 1999). Moreover, the target genes of the PcG genes may be inhibited when *MEDEA* expression is increased resulting in normal development of seeds, and this phenomenon explains the expression of *MEDEA* genes as the major effect of triploid block of seeds (Erilova *et al*., 2009).

4.1.4. Triploidy and the unreduced gametes

Many important crop plants are polyploids (Otto and Whitton, 2000). However, even plants with small genomes, such as *A. thaliana,* are paleopolyploids (Blanc *et al.*, 2000; Adams and Wendel, 2005 and d'Erfurth *et al.,* 2008). For a long time, polyploids were thought to originate from somatic chromosome doubling (Wingë, 1917). Triploids can form by fusion of an unreduced (2n) gamete with a haploid gamete or by polyspermy. Equational division of the female meiocyte in dyads indicated the likelihood of fertilisation of unreduced female gametes by haploid male gametes (Ravi *et al*., 2008). In this case, the new developed seeds would carry an excess contribution from the maternal genome.The most common meiotic abnormalities that lead to the formation of (2n) gametes include abnormal cytokinesis, the omission of the first or second division and abnormal spindle arrangement, in particular parallel or fused spindles (co-orientation of second division spindles), (Veilleux, 1985; Bretagnolle and Thompson, 1995; d'Erfurth *et al.,* 2008). The frequency of unreduced gametes in diploid plants can approach a few percent according to the species, genotype, and environmental conditions such as temperature and chemical agents (Carputo *et al.*, 2003; Ramsey, 2006; Kato and Birchler, 2006; Dilkes *et al.*, 2008). However, the production of 2n gametes is under strong genetic control (Ramsey and Schemske, 1998).

The fusion of both normal and unreduced gametes can result in the formation of tetraploid plants. In some species without strong postzygotic lethality interploidy crosses can form polyploids via the production of the triploid bridge (Ramsey and Schemske 1998: Dilkes *et al.*, 2008). These triploids serve as a bridge or step towards higher ploidy levels (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998; Otto and Whitton, 2000 and d'Erfurth *et al.,* 2008).

4.1.5. Triploid block in the ecotype Col-0

Scott *et al*., (1998a) tested only two ecotypes, L*er* and C24, in [2x X 4x] or [4x X 2x] crosses, making any generalizations about triploid block in *A. thaliana* impossible. Ecotypes are different land races of the same species of plant (Crossa Raynaud, 1977); however, *A. thaliana* has many different ecotypes (over 500,000 ecotypes) that are widely distributed and grow in differing climates across the world. Ecotypes are a valuable source of with-in species genetic variation, and has been exploited to identify genes and quantitative trait loci that underpin complex phenotypes such as Col-0/L*er* (Lister and

Dean, 1993; Meyre *et al*., 2001), C24/Col-0 (Törjek *et al*., 2006, 2008; Lisec *et al*., 2009), Cvi-0/L*er* (El-Assal *et al*., 2001), Col-0/L*er*/Cvi-0 (Alonso-Blanco *et al*., 1998), and Kas-1/Tsu-1 (McKay *et al*., 2008; Juenger *et al*., 2010).

Dilkes *et al*., (2008) working with the Col-0 ecotype first demonstrated the existence of genetic variation for triploid block in *A. thaliana.* In Col-0, in contrast to L*er* and C24, the [2x X 4x] cross exhibited high levels of triploid block; the reciprocal [4x X 2x] cross behaved as L*er* and C24 in being non-abortive. Such an extreme asymmetric or paternal-only, postzygotic hybridisation barrier had not been reported previously in plants. An important question that the work reported in this thesis set out to address was which of the two behaviors - no triploid block or paternal-only triploid block - or some other permutation, is most representative of the *A. thaliana* species. The experimental approach was to test the crossing behaviour of multiple additional ecotypes and to use meiotic mutants to investigate aspects of the Col paternal- only triploid block.

4.2. Results

4.2.1. The Columbia syndrome

Scott *et al*., (1998a) showed that reciprocal [2x X 4x] and [4x X 2x] interploidy crosses in the *A. thaliana* ecotypes L*er* and C24 resulted in very low levels of seed lethality (Table 4.1). In the present work, these same crosses were repeated with the addition of the Columbia (Col-0) ecotype. The crosses between tetraploid 4x seed parent and diploid 2x pollen parent in these ecotypes again produced mainly normal plump seeds with low frequency of abortion (Table 4.1)

In contrast, whilst the L*er* and C24 [2x X 4x] crosses again resulted in low levels of seed abortion, the Col-0 cross was highly abortive, with 89.2 % ± 1.4 of the seed shrivelled (Table 4.1; Figures 4.1 and 4.2). During the course of the present work, Dilkes *et al.*, (2008) published the results of Col-0 interploidy crosses which were similar to those described above: 75-90% shrivelled seed for the [2x X 4x] cross and 3-4 % for the [4x X 2x] cross. These data reveal that the Col-0 ecotype exhibits an asymmetric triploid block, where a [4x X 2x] cross is non-abortive, but the reciprocal [2x X 4x] cross is highly abortive. This is termed 'paternal triploid block'.

Table 4.1: Outcome of balanced and both [2x X 4x] and [4x X 2x] interploidy crosses in the ecotypes Col-0, L*er*, and C24.

	Seed parent	Pollen parent	Predicted embryo ploidy (m:p)	Predicted endosperm ploidy (m:p)	Frequency of shrivelled seeds (%)		
					Col-0	L*er*	C24
Balanced cross	2x	2x	2x (1:1)	3x (2:1)	0	0	0
Maternal excess	4x	2x	3x (2:1)	5x (4:1)	5.8 ± 2.9	11.6 ± 3.8	0.2 ± 0.0
Paternal excess	2x	4x	3x (1:2)	4x (2:2)	89.2 ± 1.4	15.8 ± 3.0	5.3 ± 0.5

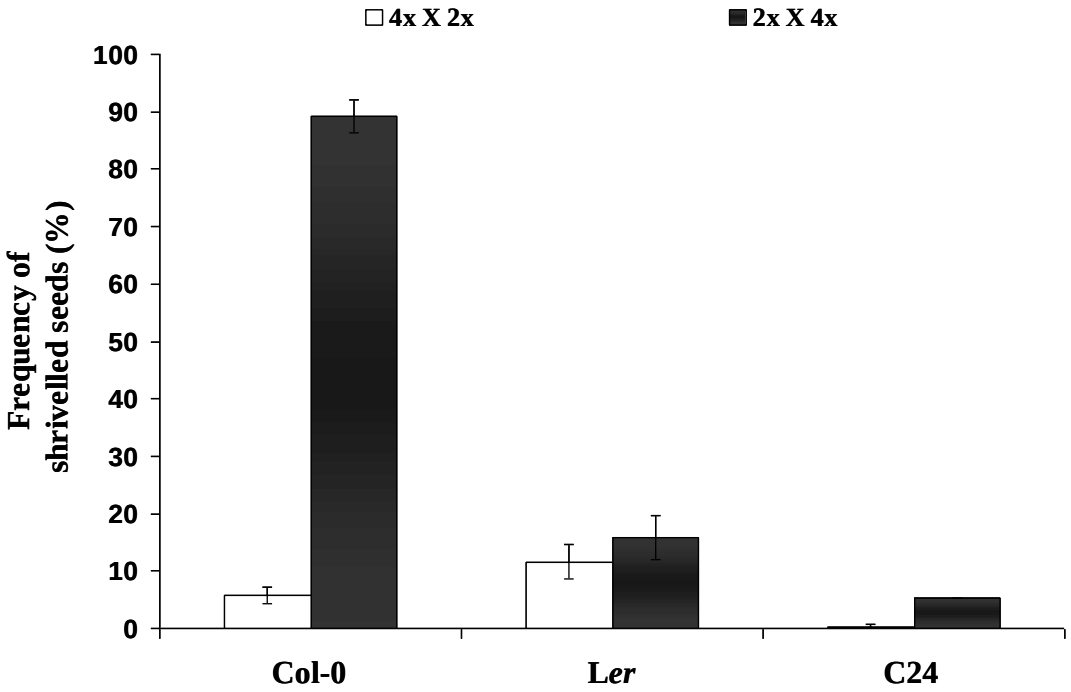

Figure 4.1: Frequency of shrivelled seeds in reciprocal interploidy crosses [2x X 4x and 4x X 2x] in the *A. thaliana* ecotypes Col-0, L*er*, and C24

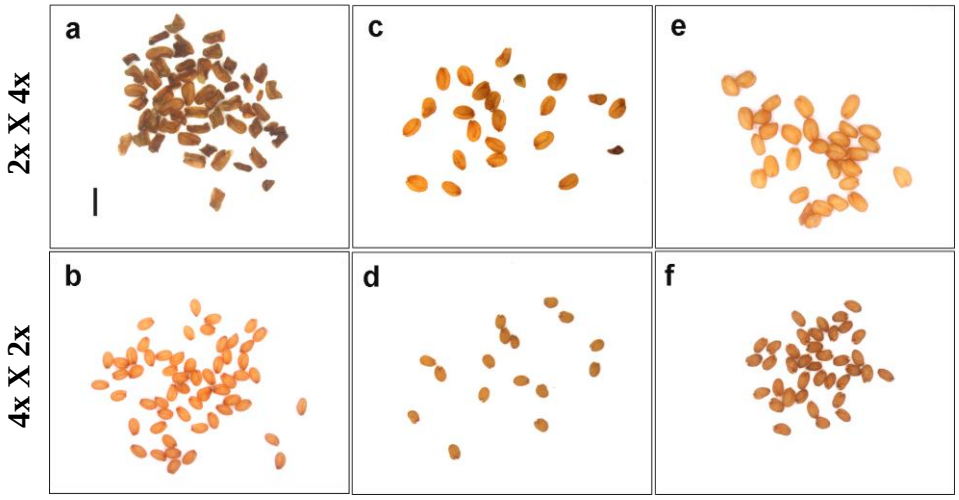

Figure 4.2: Seeds produced following reciprocal interploidy crosses in Col-0, L*er*, and C24 ecotypes. Images show mature seeds, (a,b) Col-0 (c,d) L*er* (e,f) C24. Scale bar= 1mm

4.2.2. Genetic variation for asymmetric interploidy hybridisation barriers

Having established that Col-0 behaves differently to L*er* and C24 in possessing paternal triploid block, an important question was which of these two behaviors is most representative of *A. thaliana*. To address the question, autotetraploid plants of a further eleven *A. thaliana* ecotypes (Table 4.2) were generated by colchicine treatment from diploid stocks sourced from NASC for use in reciprocal interploidy crosses. Putative tetraploids were identified based on a large flower size relative to untreated diploid plants, and subsequently verified as tetraploid by karyotyping. Reciprocal intra-ecotype interploidy crosses were then conducted and the outcomes assessed using two measures: 1) the frequency of shrivelled seed and 2) the mean seed weight. The mean seed weight was calculated from the total number of seeds per silique (shrivelled and plump) and the total weight of these seeds (shrivelled and plump), and was intended to provide a more objective measure of seed abortion than the potentially subjective measure of shrivelled seed frequency.

Table 4.2: Information on the origin of 14 *A. thaliana* ecotypes used in this project
(N= number of the stock as shown in NASC)

Ecotype	Location	Altitude (meters)	Longitude (min-max)	Latitude (min-max)	Spring daily temp. (° C)	Autumn daily temp. (° C)	Height (cm)	Mean weight of (2xX2x) seeds/silique µg (number)	Habitat
Col-0 (N1093)	Direct descendant of Col-1	unknown	unknown	unknown	15-16	21-22	15-24	16.9 (56)	unknown
L*er* (NW20)	Mutant produced at Wageningen University	unknown	unknown	unknown	unknown	unknown	not given	18.9 (52)	unknown
C24 (N906)	The Donor is Syngenta AG Brigitte Dammn	unknown	unknown	unknown	unknown	unknown	5-30	26.9 (65)	unknown
Tsu-0 (N1564)	Tsu (Japan)	1-100	E136-E137	N34-N35	9-10	19-20	32-39	26.2 (56)	unknown
Cvi-0 (N1096)	Cape-Verdi Islands	1200	W23-W25	N15-N17	unknown	unknown	16-25	32.5 (57)	Rocky wall with moss
Kas-1 (N1264)	Kashmir (India)	1580	E74-E80	N34-N36	unknown	unknown	not given	31.2 (55)	Disturbed site
Bur-0 (N1028)	Burren (Eire)	unknown	W6-W10	E52-E55	unknown	unknown	7-30	25.7 (52)	Wall by road side
Per-1 (N1444)	Perm (USSR)	1-100	E56	N58	unknown	unknown	37-44	20.8 (53)	unknown
Stw-0 (1538)	Stobowa/Orel (USSR)	100-200	E36-E37	N52-N53	0-2	7-8	31-45	20.3 (50)	Sandy fallow land
Co-2 (N1086)	Coimbre (Portugal)	100-200	W8-W9	N40-N41	11-12	17-18	16-26	21.1 (48)	Botanic garden
Ob-0 (N1418)	Oberursel/Hasen (Germany)	100-200	E8-E9	N50-N51	4-5	9-10	32-42	22.1 (50)	Roadside sandy loam
Ws-0 (N1602)	Vaseljevisi (Wassilewskija)/ Dnjepr (USSR)	100-200	E30	N52-N53	3-4	5-6	33-38	20.5 (56)	Sandy ryefield
Bla-0 (N970)	Blanes/Gerona (Spain)	100	E3	N41-N42	17-18	11-12	25-37	22.8 (54)	unknown
RLD (N28687)	University of Chicago (USA)	unknown	unknown	unknown	unknown	unknown	not given	19.9 (53)	unknown

4.2.2.1. Intra-ecotype [2x X 4x] crosses

The intra-ecotype [2x X 4x] crosses featuring the newly generated autotetraploids resulted in one of two outcomes with respect to seed development: either L*er*/C24-like (10 from 11 ecotypes) or Col-0-like (1 from 11 ecotypes). The L*er*/C24-like group was characterised by a low frequency of shrivelled seed (0.9 - 31.4%) and a high average seed weight (26.4 - 41.9 µg), similar to the L*er* and C24 control crosses (Figure 4.3; Table 4.3). Bla-1 showed the lowest frequency of shrivelled seeds (0.9 %; Figure 4.4a) and as expected this was associated with a relatively high mean seed weight (28.3 µg). Kas-1 displayed the highest frequency of shrivelled seed (31.4%; Figure 4.4b); however the mean

seed weight was relatively high (33.2 μg). Other ecotypes such as Ob-0 showed an intermediate frequency of shrivelled seeds (10.1 %; Figure 4.4c); while the mean seed weight was 26.6 μg.

The Col-0-like group, which consisted of the single ecotype RLD, was characterised by a high frequency of shrivelled seed (89.2%; Figure 4.4d) and a low average seed weight (12.0 μg), similar to Col-0.

This data indicates that majority of *A. thaliana* ecotypes are resistant to their autotetraploids in [2x X 4x] crosses, and that only a minority of ecotypes (Col-0 and RLD) display paternal triploid block.

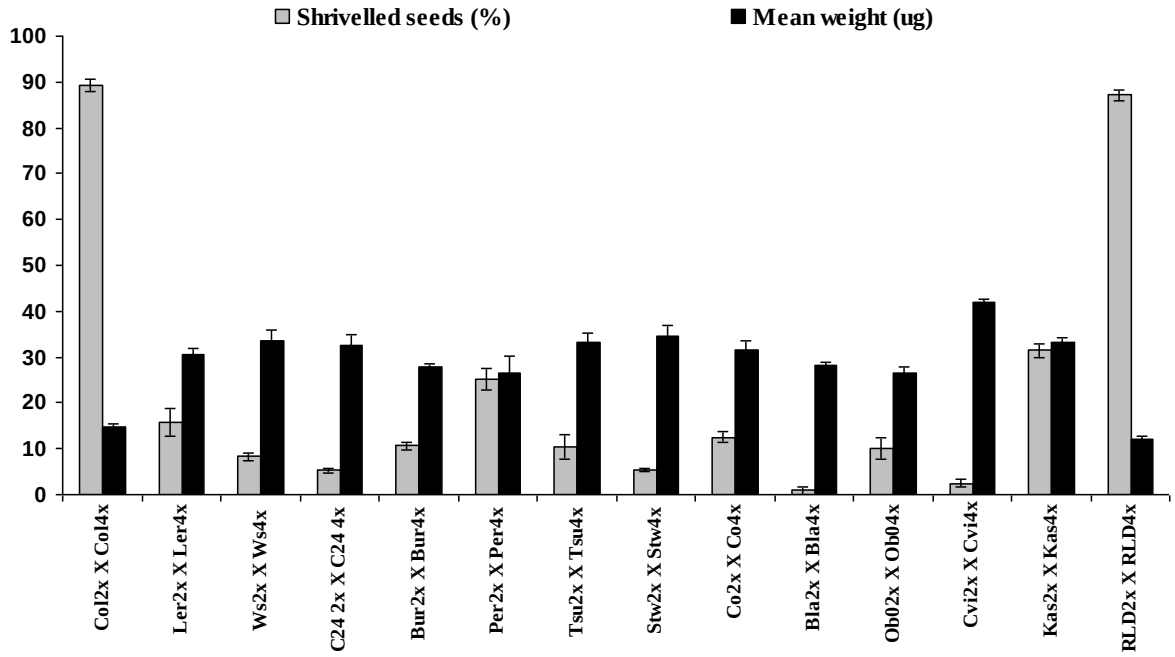

Figure 4.3: Genetic background determines the level of abnormal seed development in intra-ecotype interploidy [2xX4x] crosses. The histogram shows the frequency of shrivelled seeds and the individual seed weight following intra-ecotype interploidy crosses in 14 different *A. thaliana* ecotypes. Error bar represents standard error of the mean.

Table 4.3: Quantifying the impact on seed development of in intra-ecotype [2xX4x] crosses in *A. thaliana*. Two measures were used: frequency of shrivelled seeds and the mean individual seed weight. se = standard error of the mean.

Cross	Shrivelled seeds (%) ± se n= (seeds/silique)	Mean weight (µg) ± se n= (seeds/silique)
Col2x X Col4x	89.2 ± 1.4 (792/20)	14.9 ± 0.6 (792/20)
Ler2x X Ler4x	15.8 ± 3 (236/6)	30.6 ± 1.2 (236/6)
Ws2x X Ws4x	8.3 ± 0.8 (345/8)	33.5 ± 2.3 (345/8)
C24 2x X C24 4x	5.3 ± 0.5 (276/5)	32.7 ± 2.3 (276/5)
Bur2x XBur4x	10.6 ± 0.9 (342/7)	27.9 ± 0.6 (342/7)
Per2x X Per4x	25.1 ± 2.4 (243/6)	26.4 ± 3.9 (243/6)
Tsu2x X Tsu4x	10.4 ± 2.7 (304/7)	33.3 ± 2 (304/7)
Stw2x X Stw4x	5.4 ± 0.2 (298/6)	34.4 ± 2.4 (298/6)
Co2x X Co4x	12.5 ± 1.1 (237/6)	31.6 ± 1.9 (237/6)
Bla2x X Bla4x	0.9 ± 0.9 (109/3)	28.3 ± 0.7 (147/4)
Ob-02x X Ob-04x	10.1 ± 2.3 (194/4)	26.6 ± 1.1 (194/4)
Cvi2x X Cvi4x	2.4 ± 0.8 (183/6)	41.9 ± 0.7 (107/3)
Kas2x X Kas4x	31.4 ± 1.4 (180/6)	33.2 ± 0.9 (105/3)
RLD2x X RLD4x	87.1 ± 1.1 (403/8)	12.0 ± 0.6 (233/5)

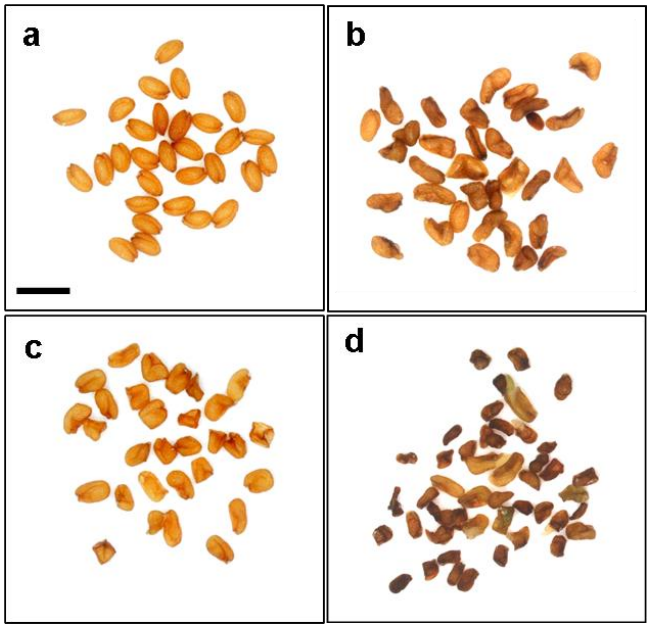

Figure 4.4: Seeds produced following intra-ecotype interploidy [2xX4x] crosses showing ecotypic variation in the frequency of abnormal development (low, moderate and high). Images show mature seeds. (a) Bla-0 (b) Kas-1 (c) Ob-0 (d) RLD. Scale bar = 1mm.

4.2.2.2. Intra-ecotype [4x X2x] crosses

Although Col-0 displays paternal triploid block, [4x X 2x] crosses have very low levels of seed abortion (Dilkes *et al.*, 2008) that are similar to L*er* and C24 (Scott *et al.*, 1998a). To test whether 1) the absence of maternal triploid block represents the general case in *A. thaliana* and 2) RLD behaves like Col-0 in having paternal-only triploid block, the group of newly produced autotetraploids were subjected to intra-ecotype [4x X 2x] crosses. The data shown in Figure 4.5 and Table 4.4 shows that the frequency of shrivelled seed was relatively low (0.2-11.4%) across all the ecotypes, including RLD. Mean seed weights were also similar across all ecotypes. These data indicate that the crosses were non-abortive.

C24 displayed the lowest frequency of shrivelled seeds (0.2 %; Figure 4.6a) whilst Per-1 had the highest frequency of shrivelled seeds (13.0 %; Figure 4.6b). There appeared little correlation between the frequency of shrivelled seeds and the mean seed weight. For example, the frequency of shrivelled seed in Kas-1 was relatively high (11.4 %) whilst the mean seed weight was also relatively high (18.5 µg) compared to other ecotypes (Figure 4.6c). Cvi-0 had the highest mean seed weight (20.1 µg) value and a low frequency of shrivelled seeds (5.0 %; Figure 4.6d).

The above data shows maternal triploid block does not occur within the 14 ecotypes tested, indicating that *A. thaliana* may not possess maternal triploid block. The data also shows that RLD, like Col-0 has paternal-only triploid block.

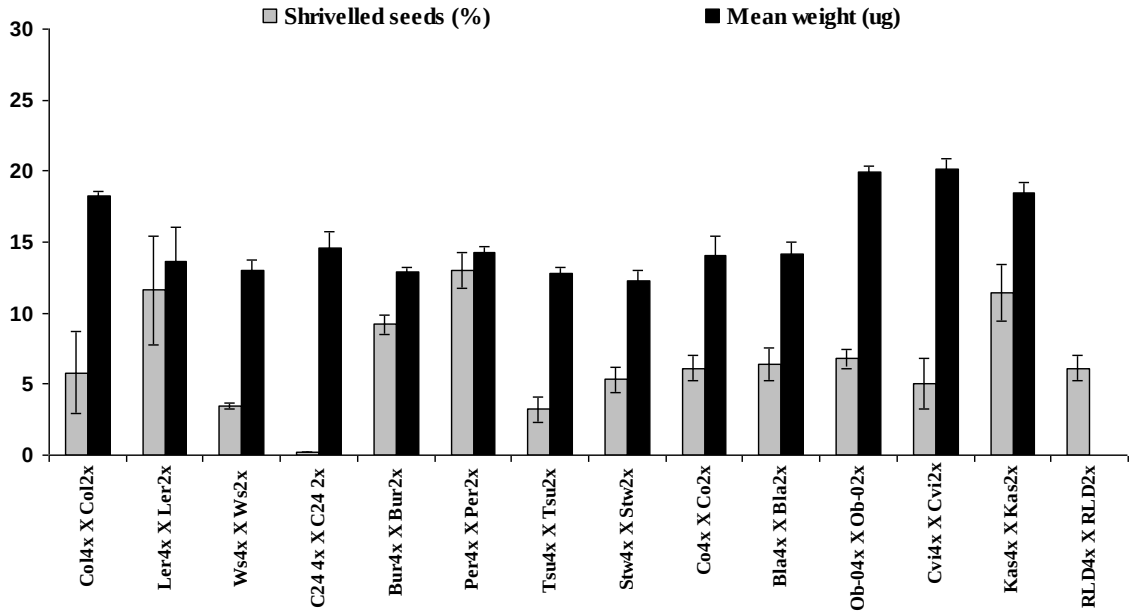

Figure 4.5: Genetic background determines the level of abnormal seed development in intra-ecotype interploidy [4xX2x] crosses. The histogram shows the frequency of shrivelled seeds and the individual seed weight following intra-ecotype interploidy crosses in 14 different *A. thaliana* ecotypes. Error bar represents standard error of the mean.

Table 4.4: Quantifying the impact on seed development of in intra-ecotype [4xX2x] crosses in *A. thaliana*. Two measures were used: frequency of shrivelled seeds and the mean individual seed weight. se = standard error of the mean.

Cross	Shrivelled seeds (%) ± se n= (seeds/silique)	Mean weight (µg) ± se n= (seeds/silique)
Col4x X Col2x	5.8 ± 2.9 (205/4)	18.2 ± 0.4 (205/4)
Ler4x X Ler2x	11.6 ± 3.8 (199/5)	13.6 ± 2.5 (199/5)
Ws4x X Ws2x	3.5 ± 0.2 (261/6)	13.0 ± 0.7 (261/6)
C24 4x X C24 2x	0.2 ± 0 (210/5)	14.6 ± 1.1 (210/5)
Bur4x X Bur2x	9.2 ± 0.7 (234/5)	12.9 ± 0.3 (234/5)
Per4x X Per2x	13.0 ± 1.3 (232/7)	14.3 ± 0.4 (232/7)
Tsu4x X Tsu2x	3.2 ± 0.9 (222/6)	12.8 ± 0.4 (222/6)
Stw4x X Stw2x	5.3 ± 0.9 (198/5)	12.3 ± 0.7 (198/5)
Co4x X Co2x	6.1 ± 0.9 (211/6)	14.1 ± 1.3 (211/6)
Bla4x X Bla2x	6.4 ± 1.2 (236/6)	14.2 ± 0.8 (236/6)
Ob-04x X Ob-02x	6.8 ± 0.7 (143/4)	19.9 ± 0.4 (143/4)
Cvi4x X Cvi2x	5.0 ± 1.8 (71/4)	20.1 ± 0.8 (63/3)
Kas4x X Kas2x	11.4 ± 2 (108/3)	18.5 ± 0.7 (108/3)
RLD4x X RLD2x	6.1 ± 0.9 (166/4)	ND ND

ND= Not done

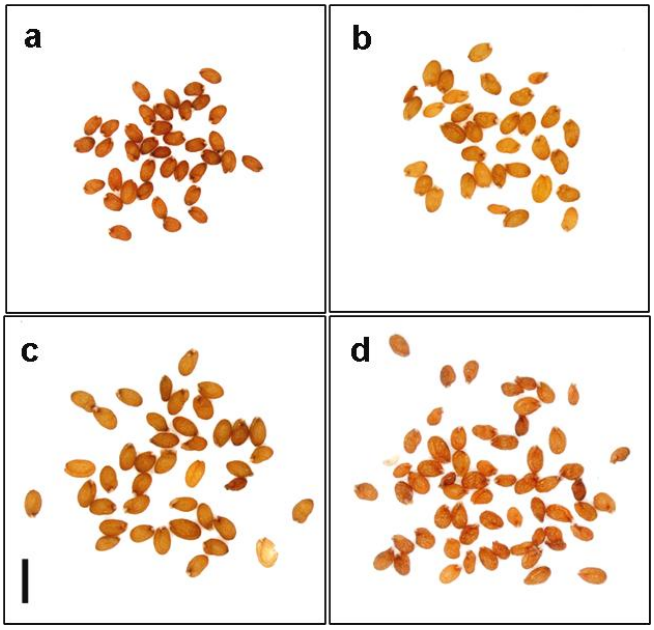

Figure 4.6: Seeds produced following intra-ecotype interploidy [4xX2x] crosses showing ecotypic variation in the frequency of abnormal development (low, moderate and high). Images show mature seeds. (a) C24 (b) Per-1 (c) Kas-1 (d) Cvi-0. Scale bar = 1mm

4.2.3. Weak mother or strong father?

Having established that Col-0 displays an unusual asymmetric paternal-only triploid block, the next objective was to investigate the underlying cause of this behaviour. In *A. thaliana*, [2x x 4x] interploidy crosses result in endosperm over-proliferation which can lead to the production of large viable seed (C24 and L*er*) or to seed abortion (Col-0) (Scott *et al*., 1998a; Dilkes *et al*., 2008). It was reasoned that this different response to paternal genomic excess could result from ecotype variation in either the 2x seed parent or the derived 4x pollen parent. For example, C24 and L*er* could be characterised as 'resisting' their respective autotetraploid, and Col-0 as 'submissive' to its autotetraploid. In turn, the inability of Col-0 to 'resist' its autotetraploid could be asymmetric in nature: the seed parent may mount a normal level of resistance to over-proliferation, but the 4x pollen parent promotes unusually high levels of over-proliferation; or the 4x pollen parent is normal in its capacity to promote endosperm over-proliferation, but the seed parent has a reduced ability to constrain proliferation. The following experiments test this 'weak' mother or 'strong' father proposition.

4.2.3.1. Inter-ecotype [Col 2x X ecotype 4x] crosses

To test whether Col-0 represents a 'weak' mother as outlined above, interploidy crosses were performed between 2x Col-0 seed parents and autotetraploid pollen parents of 13 ecotypes: Col-0, L*er*, C24, Bur-0, Per-1, Tsu-0, Stw-0, Co-0, Bla-1, Ob-0, Cvi-0, Kas-1, and RLD (Table 4.2). The effect of each cross on seed development was assessed by measuring the frequency of shrivelled seed and the average seed weight (Figure 4.7: Table 4.5).

Although there was considerable ecotype variation for both these parameters, the data identified two clear ecotype groups. The first group, which represented 11 ecotypes (L*er*, C24, Bur-0, Per-1, Tsu-0, Stw-0, Co-0, Bla-1, Ob-0, Cvi-0, Kas-1) displayed a low to moderate impact on seed development relative to the control Col-0 cross, with the frequency of shrivelled seed in the range 0.4- 40.3 %, and mean seed weights in the range 15.8- 40.3µg. Bla-1 showed the lowest frequency of shrivelled seeds (0.4 %) and had a high mean seed weight (30.7 µg; Figure 4.8a). Other ecotypes such as Stw-0 showed had a higher frequency of shrivelled seed (15.8 %; Figure 4.8b), and maintained a high mean seed weight (32.6 µg; Figure 4.8b). Crosses of 2x Col-0 to both 4x Bur-0 and 4x Tsu-0 resulted in the largest impact on seed development within this group, with a frequency of shrivelled seed of 30.4 % and 40.6 %, respectively. The mean seed weight of the Tsu-0 cross was 15.8 µg (Figure 4.8c) and Bur-0, 28.6 µg.

The second group, which contained the single ecotype RLD, had a large impact on seed development (Figure 4.8d) comparable to the control [2x X 4x] Col-0 cross. For the RLD cross, the frequency of shrivelled seed was 78.6 % and the mean seed weight 19.9 µg. This compares to 89.2 % and 14.9 µg for the Col-0 cross.

The above data shows that a 2x Col-0 seed parent resists tetraploid pollen parents from the majority of ecotypes. The only exception was RLD, which as shown earlier exhibits similar behaviour to Col-0 in reciprocal intra-ecotype interploidy crosses. The relationship between Col-0 and RLD is examined in more detail below.

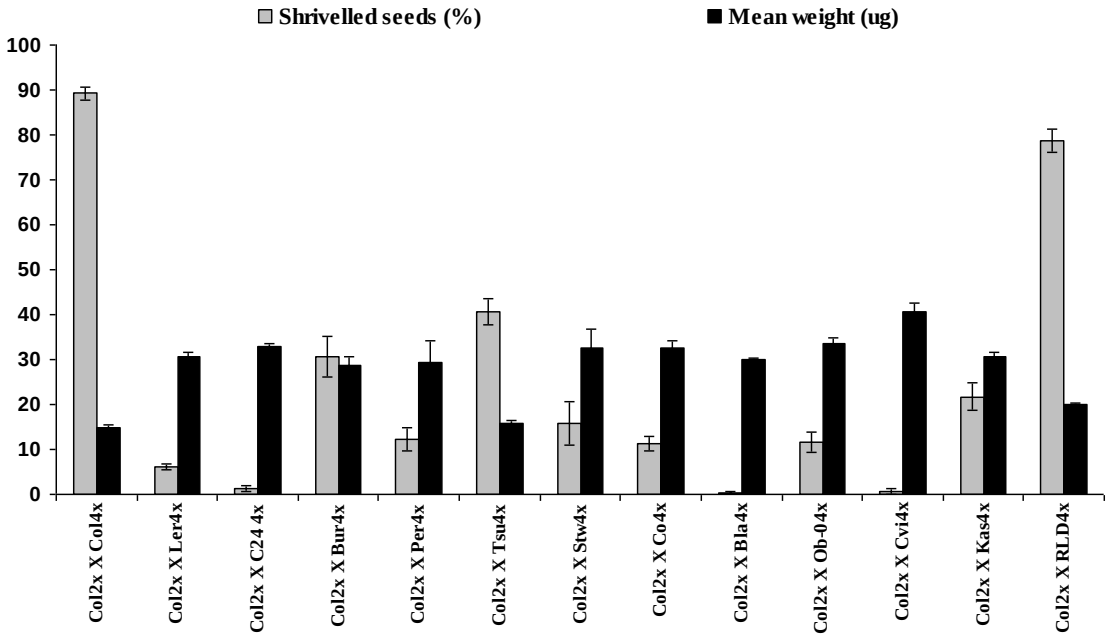

Figure 4.7: Genetic background determines the level of abnormal seed development in inter-ecotype interploidy [Col2xX4x ecotype] crosses. The histogram shows the frequency of shrivelled seeds and the individual seed weight following inter-ecotype interploidy crosses in 14 different *A. thaliana* ecotypes. Error bar represents standard error of the mean.

Table 4.5: Quantifying the impact on seed development of in inter-ecotype [Col2xX4x ecotype] crosses in *A. thaliana*. Two measures were used: frequency of shrivelled seeds and the mean individual seed weight. se = standard error of the mean.

Cross	Shrivelled seeds (%) ± se n= (seeds/silique)	Mean weight (µg) ± se n= (seeds/silique)
Col2x X Col4x	89.2 ± 1.4 (792/20)	14.9 ± 0.6 (792/20)
Col2x X Ler 4x	6.1 ± 0.5 (362/10)	30.6 ± 1.1 (158/5)
Col2x X Ws4x	ND ND	ND ND
Col2x X C24 4x	1.3 ± 0.6 (392/5)	32.8 ± 0.7 (196/5)
Col2x X Bur4x	30.7 ± 4.5 (325/10)	28.6 ± 2.2 (140/5)
Col2x X Per4x	12.3 ± 2.5 (294/10)	29.2 ± 4.9 (127/5)
Col2x X Tsu4x	40.6 ± 2.8 (376/10)	15.8 ± 0.6 (183/5)
Col2x X Stw4x	15.8 ± 4.7 (429/11)	32.6 ± 4.2 (102/3)
Col2x X Co4x	11.3 ± 1.5 (569/16)	32.6 ± 1.5 (189/5)
Col2x X Bla4x	0.4 ± 0.4 (222/4)	30.0 ± 0.2 (222/4)
Col2x X Ob-04x	11.6 ± 2.2 (526/12)	33.7 ± 1.1 (141/3)
Col2x X Cvi4x	0.7 ± 0.7 (122/4)	40.5 ± 2 (101/5)
Col2x X Kas4x	21.7 ± 3 (515/11)	30.7 ± 0.8 (227/4)
Col2x X RLD4x	78.6 ± 2.6 (370/10)	19.9 ± 0.4 (163/5)

ND= Not done

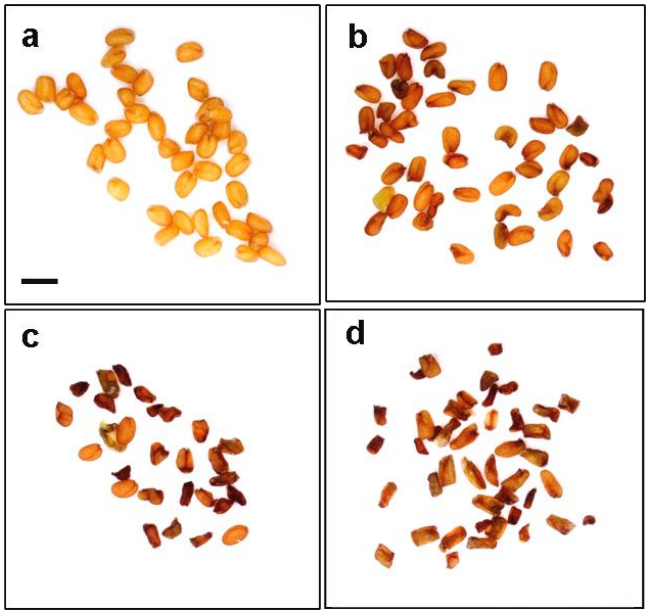

Figure 4.8: Seeds produced following inter-ecotype interploidy [Col2xX4x ecotype] crosses showing ecotypic variation in the frequency of abnormal development (low, moderate and high). Images show mature seeds. (a) Bla-1 (b) Stw-0 (c) Tsu-0 (d) RLD. Scale bar = 1mm

4.2.3.2. [ecotype 2x X Col 4x] crosses

Having found little support for the proposition that Col-0 is a 'weak' mother (4.2.3.1), [ecotype 2x X Col 4x] crosses were performed to test whether a 4x Col-0 pollen parent behaved as a 'strong' father. Two main outcomes were anticipated: the lethal effect of the 4x Col-0 pollen parent would either be restricted i.e. intra-ecotype, or generalised i.e. inter-ecotype. As before, the success or failure of the crosses was assessed by measuring the frequency of shrivelled seed and the mean seed weight.

As shown in Figure 4.9 and Table 4.6 the crosses resulted in almost continuous variation for the level of abnormal seed development. A total 7 of ecotypes (Ws, C24, Tsu, Co, Bla, Obo, Kas) had levels of abnormal seed development below 20%, as measured by the frequency of shrivelled seed. Two of these ecotypes, Bla-1 and Kas-1 (Figure 4.10 a, b), had very low frequencies of shrivelled seed (below 10 %) and high mean seed weights (> 30 µg). This group of ecotypes was therefore highly resistant to the 4x Col-0 pollen parent. In contrast the 4 ecotypes, Bur-0, Per-0, Cvi-0 and RLD produced very high levels of abnormal seed in [ecotype 2x X Col 4x] crosses. In the case of Cvi-0 (Figure 4.10c), the frequency of abnormal seed (94.2% shrivelled seed; 13.5 µg mean seed weight) was higher than the control [2x Col-0 X 4x Col-0] cross (89.2% shrivelled seed; 14.9 µg mean seed

weight). These ecotypes were therefore highly sensitive to the 4x Col-0 pollen parent. Two ecotypes, L*er* (Figure 4.10d) and Stw-0, exhibited moderate levels of abnormal seed development (around 40 % shrivelled seed, and mean seed weights around 20 µg) when crossed to the 4x Col-0 pollen parent. These ecotypes were therefore moderately resistant to the 4x Col-0 pollen parent.

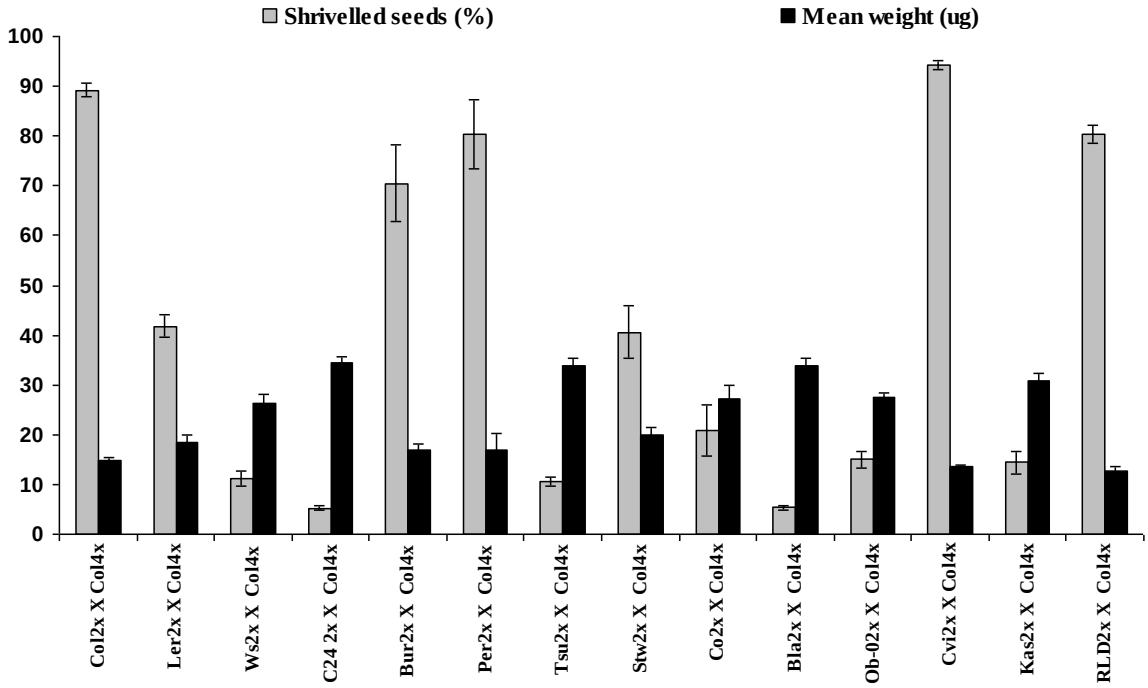

Figure 4.9: Genetic background determines the level of abnormal seed development in inter-ecotype interploidy [2x ecotypeXCol4x] crosses. The histogram shows the frequency of shrivelled seeds and the individual seed weight following inter-ecotype interploidy crosses in 14 different *A. thaliana* ecotypes. Error bar represents standard error of the mean.

Table 4.6: Quantifying the impact on seed development of in intra-ecotype [2x ecotypeX4xCol] crosses in *A. thaliana*. Two measures were used: frequency of shrivelled seeds and the mean individual seed weight. se = standard error of the mean.

Cross	Shrivelled seeds (%) ± se n= (seeds/silique)	Mean weight (µg) ± se n= (seeds/silique)
Col2x X Col4x	89.2 ± 1.4 (792/20)	14.9 ± 0.6 (792/20)
Ler2x X Col4x	41.8 ± 2.2 (315/7)	18.4 ± 1.4 (315/7)
Ws2x X Col4x	11.3 ± 1.5 (311/8)	26.3 ± 1.9 (311/8)
C24 2x X Col4x	5.2 ± 0.4 (324/7)	34.5 ± 1 (324/7)
Bur2x XCol4x	70.5 ± 7.7 (349/8)	16.8 ± 1.2 (349/8)
Per2x X Col4x	80.3 ± 6.9 (248/6)	16.9 ± 3.4 (248/6)
Tsu2x X Col4x	10.6 ± 0.8 (307/7)	33.9 ± 1.3 (307/7)
Stw2x X Col4x	40.6 ± 5.2 (327/7)	19.8 ± 1.7 (327/7)
Co2x X Col4x	20.8 ± 5.2 (376/7)	27.3 ± 2.6 (376/7)
Bla2x X Col4x	5.3 ± 0.4 (314/7)	33.8 ± 1.6 (314/7)
Ob-02x X Col4x	15.0 ± 1.7 (219/5)	27.4 ± 0.9 (219/5)
Cvi2x X Col4x	94.2 ± 0.9 (325/8)	13.5 ± 0.4 (104/3)
Kas2x X Col4x	14.4 ± 2.2 (266/6)	30.9 ± 1.3 (89/3)
RLD2x X Col4x	80.3 ± 1.8 (521/10)	12.8 ± 0.8 (245/5)

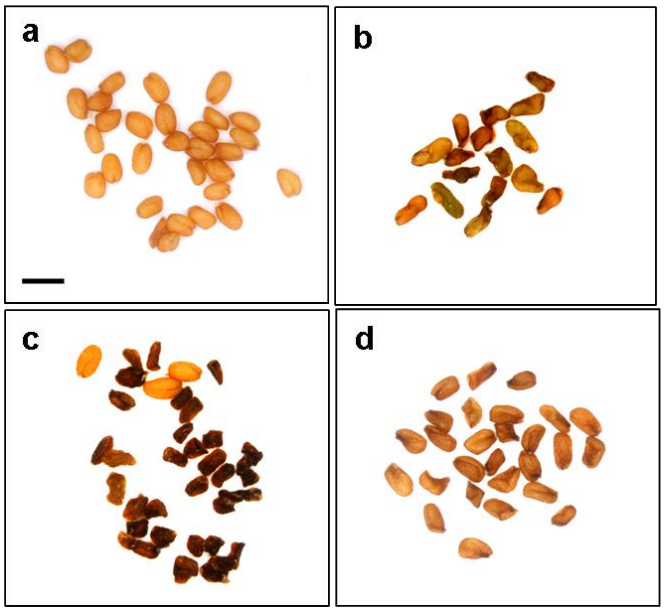

Figure 4.10: Seeds produced following inter-ecotype interploidy [2x ecotypeXCol4x] crosses showing ecotypic variation in the frequency of abnormal development (low, moderate and high). Images show mature seeds. (a) Bla-1 (b) Kas-1 (c) Cvi-0 (d) L*er*. Scale bar = 1mm

In summary, these crosses showed that the majority of ecotypes (9 from 14) were highly or moderately resistant to the 4x Col-0 pollen parent, whilst the minority (4 from 14) were highly sensitive. This data does not support the idea that 4x Col-0 acts as a universally aggressive pollen parent, but does indicate that 4x Col-0 is highly aggressive when paired with certain ecotypes.

4.2.4. The RLD ecotype has an asymmetric interploidy hybridisation barrier

The data shown in 4.2.2.1 and 4.2.2.2 established that RLD exhibits a paternal-only triploid block. The level of abnormal seed production in the [RLD 2x X RLD 4x] cross was high (87.1% shrivelled seed, 12.0 µg mean seed weight) and was low in the reciprocal [RLD 4x X RLD 2x] cross (6.1% shrivelled seed) (the relevant data is reproduced in Table 4.7, and images of mature seed are shown in Figure 4.11). These values were close to the corresponding crosses in Col-0 (Table 4.7; Figure 4.11). Besides these two ecotypes, no other ecotype of the 14 tested exhibited the behaviour. Col-0 and RLD therefore represent a relatively uncommon class of ecotypes with paternal-only triploid block. Once this was established, the interesting question arose as to whether the mechanism responsible for the behaviour in the two ecotypes had a common underlying cause. The data shown in 4.2.3.1

and 4.2.3.2 above provided some insights. Most significantly, inter-ecotype [2x X 4x] crosses between RLD and Col-0 resulted in very high levels of abnormal seed production (approximately 80 % shrivelled seed) that were similar to the intra-ecotype [2x X 4x] crosses (the relevant data is reproduced in Table 4.7; Figure 4.11). The ability of one ecotype to substitute for the other to cause high frequency seed abnormality was the first indication that the syndrome in RLD and Col-0 has a common mechanism.

Table 4.7: Quantifying the impact on seed development of in intra-ecotype [2xX4x , 4xX2x] and inter-ecotype [Col2xX4x ecotype , 2x ecotypeXCol4x] crosses in RLD *A. thaliana* ecotype. The number of seeds/ silique and se for both frequency of shrivelled seeds and mature seed weight is shown with each interploidy cross.

Cross	Shrivelled seeds (%) ± se n= (seeds/silique)	Mean weight (µg) ± se n= (seeds/silique)
RLD2x X RLD4x	87.1 ± 1.1 (403/8)	12.0 ± 0.6 (233/5)
RLD4x X RLD2x	6.1 ± 0.9 (166/4)	ND ND
RLD2x X Col4x	80.3 ± 1.8 (521/10)	12.8 ± 0.8 (245/5)
Col2x X RLD4x	78.6 ± 2.6 (370/10)	19.9 ± 0.4 (163/5)
Col2xXCol4x	89.2 ± 1.4 (792/20)	14.9 ± 0.6 (792/20)
Col4xXCol4x	0.0 ± 0.0 (137/3)	28.8 ± 0.7 (137/3)

ND= Not done

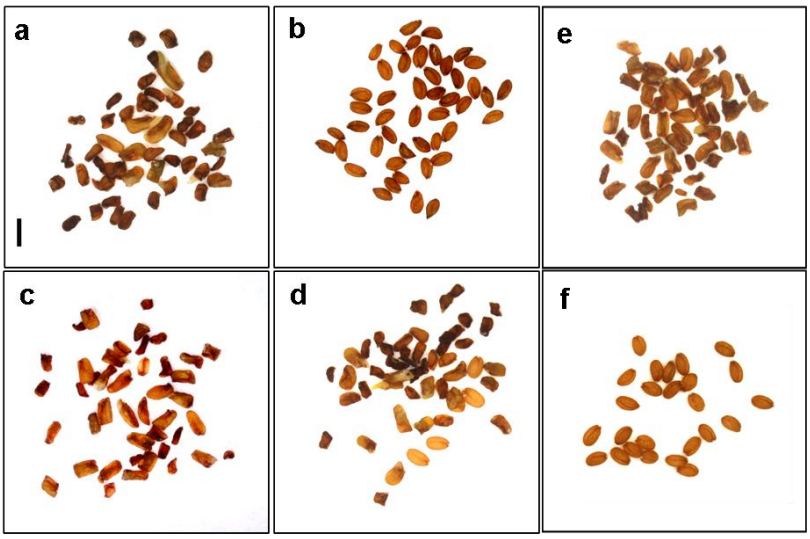

Figure 4.11: Seeds produced following inta- and inter-ecotype interploidy crosses in *A. thaliana* crosses in RLD ecotype showing the variation in abnormal development of these crosses. (a) RLD 2x X RLD 4x (b) RLD 4x X RLD 2x (c) RLD 2x X Col 4x (d) Col 2x X RLD 4x (e) Col 2x X Col4x (f) Col 4x X Col 4x. Scale bar = 1mm

4.2.4.1. The RLD and Col- 0 syndromes

To test further whether the RLD and Col-0 syndromes have a common underlying mechanism, a genetic experiment was conducted to examine the behaviour of an F_1 hybrid between 4x RLD and 4x Col-0 in [2xX4x] crosses. The rationale was that a common mechanism would most likely share a common genetic basis, whereas different mechanisms would have two different genetic origins. In a Col-0/RLD 4x hybrid, the genomes of the two ecotypes would be reduced to the diploid level, and any derived sperm would carry only a haploid complement of chromosomes for each ecotype. Importantly, crosses between RLD and Col-0 at the diploid level did not produce abnormal seed (data not shown). For a single mechanism therefore, the 4x RLD/Col-0 hybrids were expected to cause high levels of seed abnormality in [2x X 4x] crosses to either 2x seed parent (RLD or Col-0); whilst for separate mechanisms, the same crosses were expected to have much lower levels of seed abnormality.

First, two F_1 hybrids, Col-0/RLD 4x and RLD/Col-0 4x, were made by crossing 4x RLD and 4x Col-0 plants in both directions and collecting the resulting seed. F_1 hybrids plants derived from this seed were then used as pollen parents in crosses to 2x Col-0 seed parents and the frequency of plump and shrivelled seed determined in the resulting siliques (Figures 4.12 and 4.13). Control crosses between Col-0 2x and the two original RLD and Col-0 4x parents were made at the same time. The frequency of shrivelled seed in both [Col 2x X (Col/RLD 4x)] and [Col 2x X (RLD/Col 4x)] crosses was high at (77.36 %) and (90.69 %), respectively, and comparable to the control crosses, [Col 2x X Col 4x] (81.14 %) and [Col 2x X RLD 4x] (76.72 %). This data shows that haploid genomes of RLD and Col-0, combined within a diploid sperm, are able to disrupt seed development, suggesting that the RLD and Col-0 syndromes are underpinned by a common genetic mechanism.

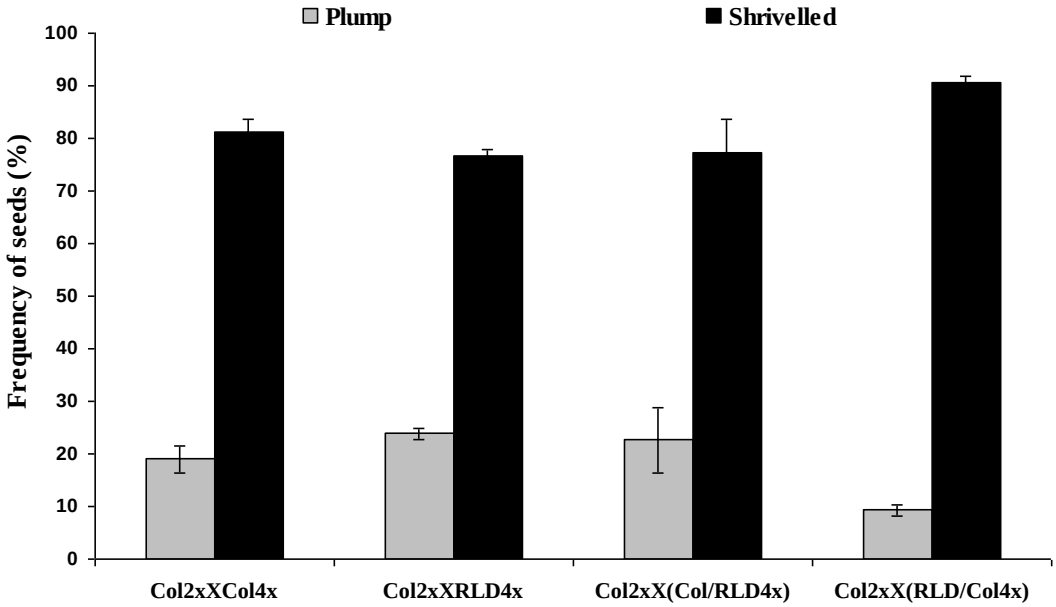

Figure 4.12: Genetic background determines the level of abnormal seed development in inter-ecotype interploidy crosses between diploid seed parent of Col-0 with pollen parents of Col 4x, RLD 4x, [Col 4x X RLD 4x], and [RLD 4x X Col 4x]. Error bar represents standard error of the mean.

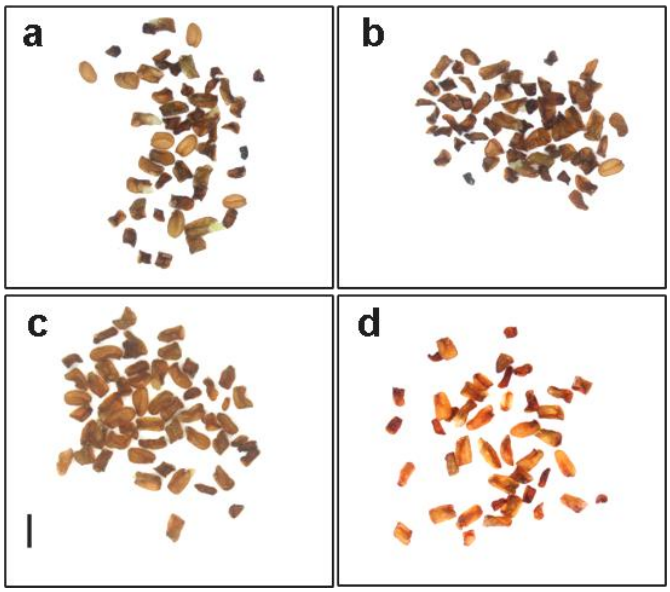

Figure 4.13: Seed produced from *A. thaliana* Col-0 and RLD interploidy crosses showing the variation in the frequency of abnormal development. (a) Col 2x X [Col/RLD 4x] (b) Col 2x X [RLD/Col 4x] (c) Col 2x X Col 4x (d) Col 2x X RLD 4x. Scale bar = 1mm.

4.2.5. Comparison between tetraploid Col-0 plants and diploid male meiotic mutants *Atps1-1* and *jas-3*

The diploid gametes of Col-0 4x plants are generated by normal meiosis from progenitor cells that have remained tetraploid through the entire life cycle of the plant. The ability of diploid sperm from 4x Col-0 plants to cause the observed high frequency of abnormal seed development in crosses to a Col-0 2x seed parent could therefore arise slowly and progressively. An alternative hypothesis was that this 'Col-killing' activity could arise rapidly following chromosome doubling, such as that occurs when unreduced gametes are formed in a diploid plant by an abnormal meiosis. The availability of *A. thaliana* meiotic mutants in the Col-0 ecotype that produce such unreduced gametes at high frequency from otherwise normal diploid plants, provided an opportunity to test whether killing activity can arise rapidly.

Two suitable mutants were *Arabidopsis parallel spindle 1* (*Atps1-1*) (d'Erfurth *et al.*, 2008) and *jason* (*jas-3*) (Erilova *et al.*, 2009). *Atps-1* and *jas-3* plants produce ~65 % and ~64 % unreduced pollen grains, respectively. The rest of the pollen is either haploid (~5 % and ~17 %, respectively) or triploid (~30 % and ~19 %, respectively). Unreduced pollen arises because aberrant (parallel) orientation of the spindle during meiosis II causes the failure of homologous chromosome to migrate to opposing poles. The remainder of pollen development is relatively normal and results in a mixture of reduced and unreduced pollen grains (d'Erfurth *et al.*, 2008 and Erilova *et al.*, 2009). Neither mutation affects female gametogenesis. Thus polyploidisation of the Col-0 genome in these mutants occurs towards the very end of the life cycle.

4.2.5.1. *Atps1-1* pollen causes high frequency seed abnormality in crosses to Col-0

In order to conduct crossing experiments, diploid *Atps1-1* homozygous mutant plants were obtained from a segregating population of F_2 plants grown from seed derived from a self-pollinated *ATPS1-1*/*atps1-1* heterozygote. *Atps1-1* homozygotes were identified by a high frequency of shrivelled seed in siliques opened at around 15 DAP and verified by examining meiosis-stage anthers for the presence of dyads (d'Erfurth *et al.*, 2008).

As shown in Figure 4.14, [Col 2x X *Atps1-1*] crosses resulted in a high frequency of abnormal seed compared to the control [Col 2x X Col 2x] cross (41.4 % ± 2.4 vs. 0.0 %;

Student's t-test, $p < 0.001$). Figure 4.14 also shows that this level of abnormal seed was 50% of that produced in the [Col 2x X Col 4x] cross. However, as outlined above, only ~65 % of pollen produced by an *Atps1-1* plant is diploid and therefore potentially capable of causing Col-killing. Thus, the frequency of diploid pollen in *Atps1-1* (~65 %) closely matched the frequency of abnormal seed (41.4 %) in the [Col 2x X *Atps1-1*] cross.

The abnormal seeds produced by the [Col 2x X *Atps1-1*] cross (Figure 4.16a) were large and irregularly shaped and morphologically similar to [Col 2x X Col 4x] seed (Figure 4.16c), suggesting that *Atps1-1* and Col 4x pollen cause abnormal seed production via the same or a similar mechanism. Since the common factor between *Atps1-1* and Col-0 4x plants is the production of diploid sperm, the data above strongly suggests that the unreduced sperm of *Atps1-1* have Col-killing activity, and that this appears rapidly in diploid sperm.

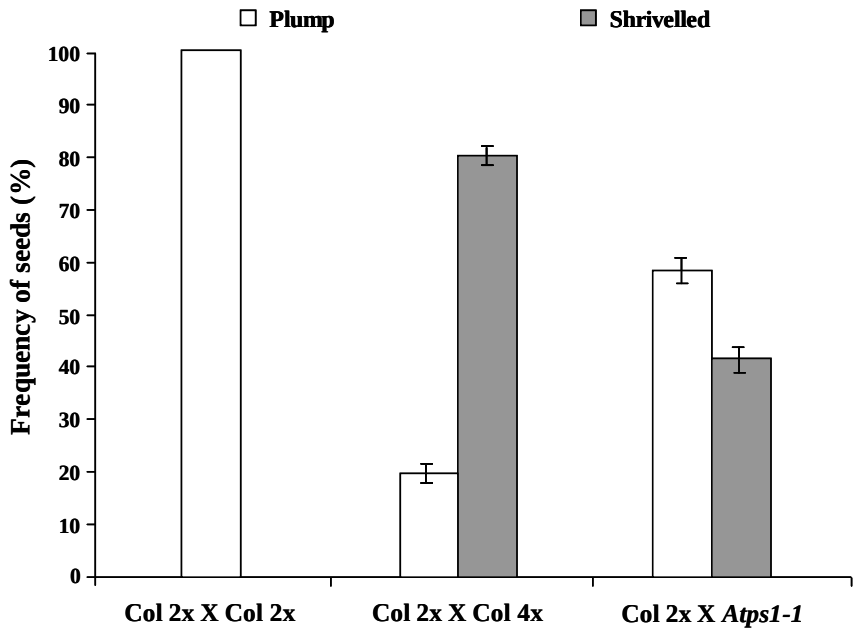

Figure 4.14: *Atps1-1* results in a high frequency of abnormal seed abortion as a pollen parent in crosses to a diploid Col-0 seed parent. n = 9 siliques
Error bar represents standard error of the mean

4.2.5.2. *jas-3* pollen also causes elevated levels of abnormal seed production in crosses with Col-0

Homozygous *jas-3* plants were produced as described for *Atps1-1* above, and used in the same series of crosses. The [Col 2x X *jas*-3] cross resulted in a significantly

increased rate of abnormal seed compared to the control [Col 2x X Col 2x] cross (24.6% ± 2.0 vs 0.0 %; Student's t-test, $p < 0.001$) (Figures 4.15 and 4.16). The rate of abnormal seed production was approximately 25 % than that of the [Col 2x X Col 4x] cross. As with *Atps1-1* only a proportion of *jas-3* pollen (~ 64 %) is reportedly diploid and the rest either haploid (~17 %) or aneuploid (~19 %) (Erilova *et al*., 2009). However, for this mutant the frequency of unreduced pollen (~ 64 %) is considerably higher than the rate of abnormal seed development. Nevertheless, the data does provide evidence that unreduced sperm formed by aberrant meiosis have Col-0 killing activity.

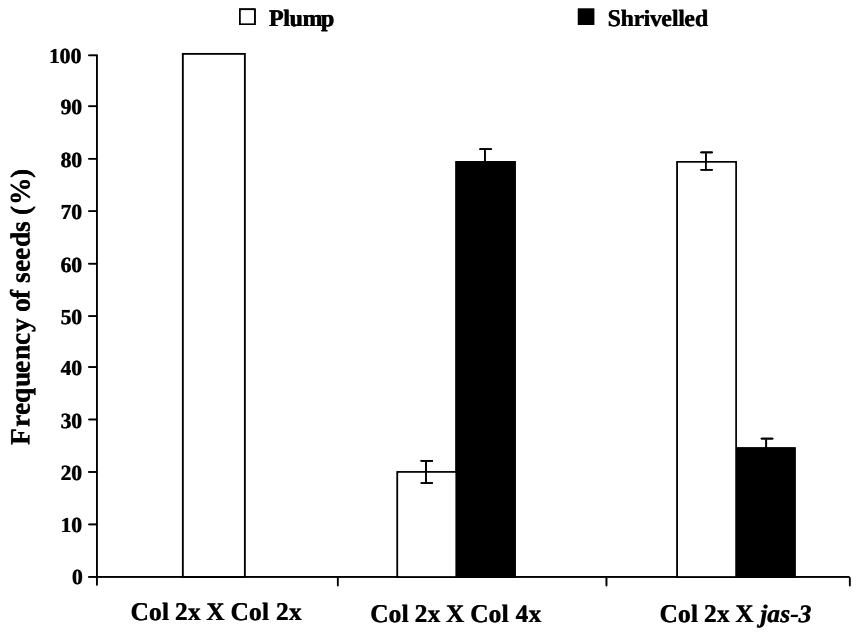

Figure 4.15: Frequency of abnormal seeds in crosses with *jas-3*.
n= 10 siliques. Error bar represents standard error of the mean.

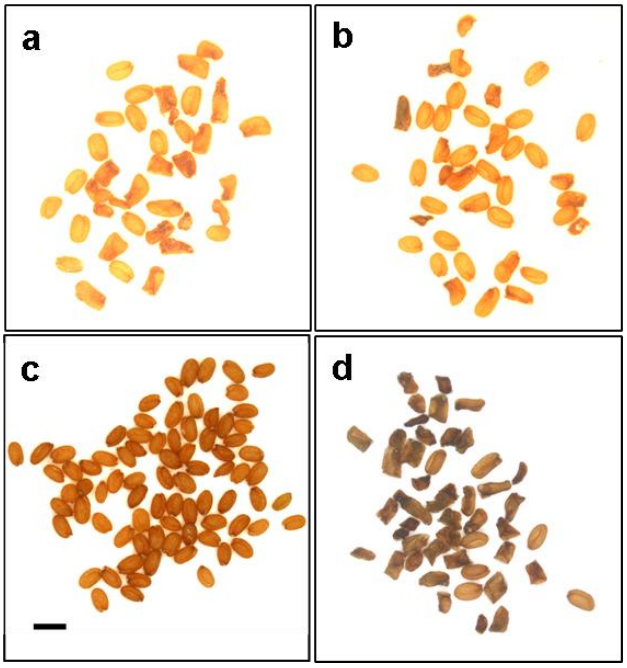

Figure 4.16: Seeds produced following the interploidy crosses between both meiotic male mutants *Atps1-1* and *jas-3* with the diploid seed parent Col-0. (a) Col 2x X *Atps1-1* (b) Col 2x X *jas-3* (c) Col 2x X Col 2x (d) Col 2x X Col 4x. Scale bar = 1mm

4.3. Discussion

Several mechanisms minimize gene flow between species and contribute to their reproductive isolation (Gutierrez-Marcos *et al*., 2003; Rieseberg and Blackman, 2010). One class of mechanism termed a postzygotic hybridisation barrier acts within the seed following fertilisation (Bushell *et al*., 2003). Soltis *et al*., (2007) stated that these barriers have been proposed as one of the main mechanisms that drive the process of speciation by shifting the ploidy of individuals. Speciation itself depends on the establishment of reproductive barriers that allow populations to diverge from each other (Josefsson *et al*., 2006). Indeed variability in postzygotic barriers or speciation mechanisms exist within the same species or between different species to provide the raw material for speciation (Ramsey and Schemske, 1998; Leblanc *et al*., 2002, and Dilkes *et al*., 2008). The work reported in this chapter represents a preliminary investigation into the genetic mechanisms responsible for variation in the lethality of interploidy crosses in *A. thaliana*. The objective was to improve our understanding of this type of postzygotic hybridisation barrier, and ultimately identify genes that underpin the phenomenon.

4.3.1. The behaviour of tetraploid Col-0

It has been shown previously that in *A. thaliana*, reciprocal crosses between diploid and tetraploid plants resulted in dramatic and reciprocal differences in seed size, but very low levels of seed lethality (Scott *et al*., 1998a). However, this study was restricted to only two ecotypes, namely C24 and L*er*. More recently, Dilkes *et al*., (2008) showed that for Col-0 this pattern was true for the [4x X 2x] cross, but the reciprocal [2x X 4x] resulted in very high levels of seed lethality (Figure 4.1). Thus Col-0 possessed what can be described as an asymmetric paternal-only postzygotic hybridisation barrier. In fact, Redei (1964) had much earlier described a similar outcome for reciprocal crosses between an *A. thaliana* diploid 'genotype' 'W' and its autotetraploid 'K-16'. In this case, the two crosses, [K-16 (4x) X W (2x)] and [W (2x) and K-16 (4x)], produced 7.4% and 97.3% abnormal (misshapen or shrivelled) seeds, respectively. George Redei (USA) confirmed the 'W' genotype as the Columbia ecotype in a personal communication to Rod Scott, University of Bath.

In cell biological terms, the different behaviours of C24, L*er* and Col-0 in [2x X4x] crosses appears to depend on endosperm cellularisation: C24 and L*er* endosperms cellularise, whereas in Col-0 cellularisation rarely occurs in a [2x X 4x] cross (Dilkes *et*

al., 2008). Excessive proliferation and failure to cellularise are common features of endosperm development in species with postzygotic hybrisation barriers, including those triggered by interploidy crosses (Jansky, 2005). Thus, [2x X 4x] crosses in Col-0 are representative of the situation in many plant species, making this ecotype potentially useful in identifying and dissecting the mechanisms underlying postzygotic barriers.

4.3.2. Parent-of-origin effects in intra and inter-ecotype crosses

Many researchers have stated that the result of both inter-ecotype and interspecific crosses can be manipulated by changing the ploidy of the crossing partners (reviewed by Köhler *et al*., 2010). The developed seeds resulting from intra-and inter-ecotype crosses discussed here incorporate genomes that differ in parent-of-origin. However, the interaction between genomes in both the seed and any resulting hybrid plant may be affected by this difference.

In interploidy crosses, seed abortion could be attributed to different factors such as: negative interaction between products of evolutionarily diverged gene sequences (Rieseberg and Carney, 1998), widespread changes to genome structure or gene expression (Rieseberg and Carney, 1998; Bushell *et al*., 2003), and imbalances between the effects of uniparentally expressed genes (genomic imprinting) (Haig and Westoby, 1991), either because of ploidy imbalance or divergence in gene expression patterns (Bushell *et al*., 2003). The analyses presented in section 4.2.2 of [2x X 4x] and [4x X 2x] crosses within 14 different *A. thaliana* ecotype, including Col-0, provided several important findings with respect to the impact of interploidy crosses on seed size and viability in this species. First, all [4x X 2x] and [2x X 4x] intra-ecotype crosses resulted in parent-of-origin effects on seed size, seed viability, or both (Tables 4.3 and 4.4). For each ecotype, [4x X 2x] seed was substantially smaller than its [2x X 2x] seed, and the frequency of shrivelled seed was low (> 15%); (Tables 4.2 and 4.4). Scott *et al*., (1998a) described similar outcomes for [4x X 2x] crosses in the ecotypes C24 and L*er*. Thus, the data strongly suggest that *A. thaliana* as a species responds to moderate maternal genomic excess (4m: 1p) in the endosperm by producing relatively small but viable seed. By extension, *A. thaliana* appears not to have maternal triploid block.

The behaviour of [2x X 4x] crosses was more complex. The majority of ecotypes displayed a typical parent-of-origin effect on seed phenotype: the seed was substantially larger than the [2x X 2x] control crosses and mostly plump. In contrast, RLD [2x X 4x] crosses resulted in the similarly high level of abnormal seed production as [2x X 4x] Col-0

crosses. As discussed above, both of these ecotypes produced low levels of abnormal seed in the reciprocal [4x X 2x] cross. Thus, RLD possesses a paternal-only triploid block similar to that observed in Col-0 (Dilkes *et al.*, 2008). Collectively then, 2 of the 14 ecotypes tested have this type of block, representing a frequency of 14% of *A. thaliana* ecotypes. Thus genetic variation for triploid block occurs in *A. thaliana*, but a larger number of ecotypes should be tested before drawing firm conclusions about prevalence.

4.3.2.1. The Col-syndrome: weak mother or aggressive father

Two scenarios were considered that might explain the Col-syndrome: either the maternal parent is weak or submissive to an otherwise normal diploid paternal parent, or the diploid genome of the paternal parent is unusually aggressive and the maternal parent normal. This 'weak' mother or 'strong' father idea was investigated in two crossing experiments (4.2.3). In the first, the 2x Col-0 seed parent was crossed to the panel of 4x ecotypes, to test whether Col-0 represents a weak mother (4.2.3.1). In the second, 4x Col-0 was used a pollen parent in crosses to the panel of 2x ecotypes, to test whether Col-0 is a strong father (4.2.3.2). The resulting data provided little support for the weak mother idea since, with one exception, the levels of shrivelled seed production were low in the [Col-0 2x X ecotype 4x] crosses. The exception was RLD, which resulted a high frequency of shrivelled seed that was comparable to a [Col 2x X Col 4x] cross. The behaviour of this ecotype is discussed further below (4.3.3).

In contrast, the crossing data did suggest that 4x Col-0 can behave as a strong father: crosses to 4 ecotypes (C24, Per, Cvi and RLD) resulted in high levels (>70%) of abnormal seed and a further 2 ecotypes (L*er* and Stw) produced moderate levels (>40%) of abnormal seed. However, 50% (7 from 14) of the ecotypes tested produced very low levels of abnormal seed. Thus, 4x Col-0 is not a universally aggressive pollen parent, but is highly aggressive when paired with certain ecotypes.

Interestingly, with the exception of RLD, the response of an ecotype to a 4x Col-0 pollen was not linked to its response to its own autotetraploid in [2x X 4x] crosses. The data presented in 4.2.2.1 clearly showed that only Col-0 and RLD [2x X 4x] intra-ecotype crosses resulted in high levels of abnormal seed. Thus, a seed parent capable of successfully resisting lethal endosperm disruption when crossed to its own autotetaploid fails to do so when crossed to 4x Col-0.

This above indicates that the interaction between Col-0 and other ecotypes is highly specific, but few clues as the nature of the underlying processes. One possibility however,

is that mis- or over-expression of one or a few genes involved in regulating endosperm development might be sufficient to cause endosperm failure. Dilkes *et al*., (2008) showed that seed abortion in [Col 2x X Col 4x] crosses was most likely caused by the absence or mistiming of endosperm cellularisation.

Köhler *et al*., (2010) showed that any change in the ratio of two maternal to one paternal genomes (2m:1p) such as [2x X 4x] cross in the endosperm might cause endosperm failure in interploidy crosses and the developed seeds usually abort. This is because the endosperm supports development of the embryo by providing nutrients and signals for its growth (Lopes and Larkins, 1993) and is important for viable seed formation (Costa, 2004). However, increasing the contributions of maternal and paternal genomes has opposite effects: 1) Increasing maternal genomes inhibits endosperm proliferation, 2) Increasing paternal genomes causes excess of the endosperm (Scott *et al*., 1998a). Moreover, there is a differential dosage effect of maternal and paternal factors on endosperm development (Haig and Westoby, 1991). Several possibilities could account for this effect, including the unbalanced maternal contribution to the endosperm (Scott *et al*., 1998a). Another explanation would be related to the genomic imprinting (Lin, 1984; Haig and Westoby, 1991) because the imprinted genes are differentially marked in the gametes, rendering different function for both maternal and paternal chromosomes (Feil and Berger, 2007).

The MADS box transcription factor *AGL62* promotes endosperm proliferation at the expense of cellularisation in *A. thaliana*, (Kang *et al*., 2008). The mis-expression of *AGL62* or a similar regulatory gene, could delay endosperm cellularisation and cause seed failure in interploidy crosses. Such mis-expression could result from general genomic incompatibility (Kang *et al*., 2008), or more specific causes such as the maternal genome failing to limit or counter the expression of genes such as *AGL62* from the paternal genome (Walia *et al*., 2009). Additionally, Dilkes and Comai, (2004) stated that postzygotic lethality of interspecies hybrids can results from differences in gene expression, copy number, or coding sequence and can be overcome by altering parental genome dosage (Josefsson *et al*., 2006).

4.3.3. Do RLD and Col-0 share the same syndrome?

As discussed earlier, RLD was the only newly tested ecotype to behave like Col-0 in reciprocal diploid-tetraploid crosses (4.2.2.1 and 4.2.2.2). This revealed that RLD has paternal-only triploid block. Once established, the question was addressed as to whether

the mechanism responsible for the behaviour in the two ecotypes had a common underlying cause (4.2.4.1).

Inter-ecotype reciprocal crosses had already established that substituting Col-0 and RLD one for the other resulted in the same outcomes as intra-ecotype crosses (4.2.3.1 and 4.2.3.2). This suggested that the syndrome in the two ecotypes has a common cause. This was subjected to a genetic test by examining the behaviour of an F_1 hybrid between 4x RLD and 4x Col-0 in [2x X 4x] crosses. Meiosis II in the hybrid would produce diploid sperm containing only a single haploid chromosome complement of the two parental ecotypes. Since haploid sperm do not affect seed development, the ability of the diploid hybrid sperm to cause high levels of abnormal seed production in crosses to 2x Col-0 would be evidence of a shared genetic basis. In the event, this was the case (4.2.4.1): the 4x RLD/Col-0 F_1 hybrids caused abnormal seed development at the roughly the same frequency as the original 4x parents. This suggests that the RLD and Col-0 syndromes are underpinned by a common genetic mechanism.

Until now there are no clear similarities in the genetic behaviour, environmental conditions, geographical location, disease resistance, etc. in both Col-0 and RLD ecotypes. However, RLD have been usefully used in some *Arabidopsis* biological researches such as disease resistance (Kwon *et al*., 2004), downy mildew resistance (Joos *et al*., 1996), and imprinting (Kinoshita *et al*., 1999).

4.3.3.1. The Col-0/RLD syndrome - potential mechanisms

In both Col-0 and RLD, [2x X 2x] and [4x X 4x] crosses produce only very low levels of shrivelled seed. Like wise [4x X 2x] crosses. However, for both ecotypes the [2x X 4x] cross results in >80% shrivelled seed. This is the Col-0/RLD syndrome: an asymmetric postzygotic hybridisation barrier. Any explanation of the syndrome must therefore account for this asymmetry, and in particular that 1) a diploid sperm is disruptive to seed development in a [2x X 4x] cross but not in a [4x X 4x] cross, 2) a haploid sperm is not disruptive, and 3) diploid maternal gametes in Col-0 do not maternalize seeds more than those of other ecotypes in [4x X 2x] crosses.

How might this asymmetry arise? Various studies have shown that polyploid formation can result in genomic rearrangements, epigenetic alterations to DNA and changes in gene expression patterns (Song *et al*., 1995; Weiss and Maluszynska, 2000; Comai *et al*., 2000; Kashkush *et al*., 2002; Osborne *et al*., 2003). The genomic differences that occur when a plant becomes a tetraploid could have different consequences for

maternal and paternal gametes, and therefore explain the behaviour of diploid gametes in Col-0 and RLD. Mega- and micro-gametogenesis are different in a several ways, including the number of mitosis prior to gamete formation (Scott and Spielman, 2006), the extent of chromatin condensation (Valencia, 1982), development of the surrounding sporophytic tissues (Grossniklaus and Schneitz, 1998; Simao *et al*., 2007), molecular and genetic basis of cell specification and differentiation (Grossniklaus and Schneitz, 1998). It is possible therefore that the polyploidisation–induced alterations to DNA structure could impact the two genders of gametes differently.

4.3.4. Chromosome doubling vs non-reduction

As discussed above, the Col/RLD syndrome might be a consequence of a sex-specific difference in the response to genomic changes that can accompany genome doubling. When tetraploids are formed by colchicine treatment, genome doubling and any associated genome changes occur in somatic cells well before the onset of gametogenesis. However, the most common route to polyploidisation, which occurs in many taxa, is via unreduced gametes that retain the somatic chromosome number (n=2n) (Ramsey and Schemske, 1998; Köhler *et al*., 2010). In these cases, diploid gametes usually result from the failure of meiosis to half the chromosome number, rather than as a consequence of doubling somatic chromosome number. In interesting question therefore, was whether the killing activity of Col/RLD sperm flows only from doubling somatic chromosome doubling, or can it also arise following a failure to reduce chromosome number? A related question was whether the mechanism acts slowly or rapidly following either trigger. The answer has implications for both understanding the mechanism underpinning the Col/RLD syndrome and postzygotic hybridisation barriers, and the role played by polyploidisation in reproduction isolation.

The *A. thaliana* (Col-0) mutants *Atps-1* and *jas-3* produce unreduced pollen grains at high frequency due to faulty meiosis II (d'Erfurth *et al.*, 2008; Erilova *et al*., 2009). Any Col-killing activity would therefore have to arise rapidly (after meiosis II) and as a consequence of failed reduction. Both mutants caused triploid block when used as pollen parents with a 2x Col-0 seed parent (4.2.5). In the case of *Atps-1*, the frequency of shrivelled seeds was almost equal to the frequency of diploid pollen grains, indicating that diploid sperm formed by non-reduction in this *Atps-1*and chromosome-doubling in a tetraploid are similarly disruptive to endosperm development. The data also shows that the disruptive activity arises very rapidly in *Atps-1*, since there are only two mitosis between

meiosis II, when non-reduction occurs, and fertilisation. However, more data is needed to determine whether the disruptive activity in tetraploids also appears rapidly or involves the same mechanism.

4.4. Conclusion

The data presented in this chapter revealed limited genetic variation among *A. thaliana* ecotypes for triploid block. From a total of 14 ecotypes only two, Col-0 and RLD, displayed triploid block, which was asymmetric in being restricted to the paternal tetraploid. Genetic analysis indicated that Col-0 and RLD triploid block probably operate through a common mechanism, since a tetraploid Col-0/RLD F_1 hybrid induced the same level of triploid block as the individual tetraploids. We focused on studying the ecotype Col-0 and discovered that a diploid Col-0 is not a' weak' mother, but that the tetraploid Col-0 is an aggressive' father since a number of ecotypes that resisted their own tetraploid succumbed to 4x Col-0. We identified variation in F_1 lethality in interploidy crosses that indicated that the maternal parents of some ecotypes suppress F_1 lethality caused by Col-0 paternal excess while others do not.

Chapter 5

5. The genetics of a maternal modifier

5.1. Introduction

5.1.1. The Tsu-1 maternal modifier of Col-killing

Many flowering plants have the ability to tolerate ploidy changes, but the offspring of parents with different ploidies levels frequently fail to survive due to highly disrupted seed development (Dilkes *et al*., 2008), more details are described in chapter 1 (section 1.3). We investigated this phenomenon in *A. thaliana* and found ecotype-based obvious variation in the viability of F_1 seed resulting from interploidy crosses, see more details in chapter 4 (section 4.2.1). The extent of postzygotic seed lethality was most striking in crosses between a diploid seed parent and a tetraploid pollen parent of the Columbia (Col-0) ecotype, (4.2.1). However, substituting the Col seed parent with one from other ecotypes revealed a high degree of variation in the levels of seed lethality (4.2.3.2). Some ecotypes behaved as Col, suffering similarly high levels of seed lethality, whereas others, such as C24 and Tsu-1 almost completely resisted Col-killing activity to produce near 100% viable seed. These ecotypes appear to possess maternal 'modifiers' that somehow resist the Col-killer. This chapter presents the further phenotypic and genetic characterisation of the Tsu-1 maternal modifier and preliminary efforts to map the gene or genes responsible.

5.1.2. *A. thaliana* is useful in studying genetics

Many important traits in plant breeding are controlled by a number of genes that show continuous variation among progeny (Takahashi *et al*., 2001). Genetic variation affects fitness in wild populations adapting to different environments, and phenotypic plasticity is expected to reflect the genetic variation. Because of naturally occurring variation among ecotypes in response to environment, *A. thaliana* has become an important tool in studying the genetics of plant growth and development (Meinke *et al.*, 1998). Genetic studies in *A. thaliana* has also benefited from the development and application of genetic tools such as an easy to use on-line genome sequence in a database that is continuously updated as more information is obtained about polymorphisms, gene

function, markers, and so on. Moreover, expanding databases of other plant species and genomes facilitate the application of model plant research to other crop species such as maize, rice, wheat, and *Brassica*. Today, the *A. thaliana* genome sequence and the associated database also facilitate the process of trait mapping (Lukowitz *et al.*, 2000), in principal enabling the identification of genes underlying complex phenotypes.

5.1.3. Principles and requirements for genetic mapping

To understand genetic mapping, the first important principle is that genes segregate during meiosis, thus allowing their analysis in the progeny (Paterson, 1996; Semagn *et al*., 2006). According to Mendel's second law the chromosomes assort randomly into gametes; thus the alleles of one gene will segregate independently of second gene (independent assortment law). Furthermore, where two genes are close to each other on the same chromosome non-random assortment occurs that indicates linkage (Semagn *et al.*, 2006). The closer the linkage, the greater the deviation from independent assortment.

To construct a genetic linkage map the following steps are necessary:

- Develop a mapping family from two genetically divergent parents and decide the sample size.
- Select suitable markers for the mapping population.
- Screen the parents for marker polymorphism, and genotype both parents and progenies.
- Perform linkage analysis using statistically-based programs.

Currently, genetic maps are constructed using different types and sizes of mapping populations, marker systems, statistical procedures, and computer packages (Ferreira *et al*., 2006). However, the efficiency of the mapping process is affected by any of these previous factors (Liu, 1998). In *A. thaliana*, which is self pollinating, a mapping family is easily produced from homozygous inbred parents. Mapping can be conducted in F_2 populations, backcrosses, recombinant inbred lines, doubled haploids, and near isogenic populations (Doerge, 2002; Collard *et al*., 2005; Semagn *et al.*, 2006).

Backcross (BC) breeding has been an important tool for plant breeders for more than 80 years, and with the relatively recent incorporation of transformation as a plant breeding tool the importance of the backcross method has increased (Briggs and Allard, 1953; Lewis and Kernodle, 2009). Backcrossing allows the plant breeder more precise control of allele frequencies than other traditional plant breeding methods. In most

backcross programs the objective is to recover the recurrent parent basically unchanged except for the introgression of the new characteristic or underlying gene or genes. In a backcrossing approach, repeating the process for six generations will produce a BC6 genome that is 99% derived from the recurrent parent (Babu *et al.*, 2004).

5.1.4. Polymorphism identification

In order to construct a linkage map it is necessary to identify DNA markers that show clear differences between the parents (polymorphic markers). Collard *et al*., (2005) showed that there are high levels of DNA polymorphism in cross-pollinating species compared to inbreeding species. Moreover, mapping in inbreeding species is facilitated by the selection of distantly related parents. After selecting suitable DNA markers they are screened across the whole mapping population including the parents in a process called marker genotyping.

In *A. thaliana,* the efficient use of genetic markers requires accurate knowledge of their map position. The first *A. thaliana* genetic maps were constructed using F_2/F_3 mapping populations (Koornneef *et al*., 1983; Chang *et al*., 1988; Alonso-Blanco *et al*., 1998). Additionally, Lister and Dean (1993) created recombinant inbred lines (RILs) which constitute permanent mapping populations because they are practically homozygous genotypes and can therefore be multiplied repeatedly enabling any laboratory to use them. The Lister-Dean RILs were derived from the Columbia and L*er* ecotypes; however, numerous other combinations have been made such as: L*er*/Cvi (Alonso-Blanco *et al*., 1998) and C24/Col-0 (Törjek *et al*., 2006).

Numerous different types of marker are used for trait and gene mapping in *A. thaliana*. Restriction Fragment Length Polymorphism (RFLP) markers are co-dominant and very reliable, but can prove laborious to use (Chang *et al*., 1988 and Alonso-Blanco *et al*., 1998). Different methods have been developed to accelerate the analysis of restriction polymorphisms such as the conversion of RFLP markers into PCR markers called CAPS (Cleaved Amplified Polymorphic Sequences) (Konieczny and Ausubel, 1993). AFLP (Amplified Fragment Length Polymorphism) was developed to detect restriction fragments by PCR amplification without knowledge of the sequence (Vos *et al*., 1995 and Alonso-Blanco *et al*., 1998). Other PCR-based markers include Random Amplified Polymorphic DNAs (RAPDs) (Reiter *et al*., 1992) and Simple Sequence Length Polymorphisms (SSLPs), (Bell and Ecker, 1994).

5.1.5. Identifying and mapping genes

Many research programs have identified and mapped genes in *A. thaliana* and in crop plants. In *A. thaliana*, examples include genes that regulate flowering-time (Clarke and Dean, 1994; Lee *et al.*, 1994; Mouradove *et al.*, 2002; Simpson and Dean, 2002; Searle and Coupland, 2004), seed size (Godwin, 2005; Schruff *et al.*, 2005) and embryo development (Patton, 1991). Other research has identified disease resistance genes (Parker, 1993) and ecology-related genes such as drought-inducible genes (Taji *et al.*, 1999) and an osmotic adjustment gene in rice (Robin *et al.*, 2003).

The further characterisation of the Tsu-1 maternal modifier, involved several steps: First, a cell biological analysis of seed and endosperm development was undertaken to understand how the Tsu-1 modifier prevents seed killing by the paternal tetraploid Col-0. Second, the genetics of the maternal modifier was analyzed by performing several backcrosses using both Tsu-1 and Col -0 as recurrent parent and analyzing the BC progeny for resistance or sensitivity to Col-killing. Third, different mapping techniques were applied to begin to identify the modifier gene or genes that resist the killing activity caused by Col-0.

5.2. Results

5.2.1. Seed development in interploidy [Tsu 2x X Col 4x] crosses

The interploidy cross [Col 2x X Col 4x] results in high frequency seed abortion (Dilkes *et al*., 2008; Chapter 4). However, seed parents of certain ecotypes, including Tsu-1, proved highly resistant to this Col-killing (Table 4.4). To understand how the Tsu-1 modifier prevents seed killing by the paternal tetraploid Col-0, a cell biological analysis of seed and endosperm development was undertaken

Endosperm development was analysed in seed produced by [Tsu 2x X Col 2x], [Tsu 2x X Col 4x], and [Col 2x X Col 4x] crosses. Embryo sac (ES) area and both chalazal endosperm size and the number of nodules were recorded. The embryo sac area refers to the cavity surrounded by the seed coat which contains the chalazal endosperm cyst and the embryo. These features have proven good indicators or proxies for the extent of any interploidy cross effect (Dilkes *et al*., 2008). Previous microscopy (DIC and confocal) studies established that 4 specific days of seed development (3, 5, 6, and 7 DAP) are optimum in detecting changes in endosperm development that are associated with the interploidy cross effect (peripheral endosperm under- or over- proliferation and the timing of endosperm cellularisation).

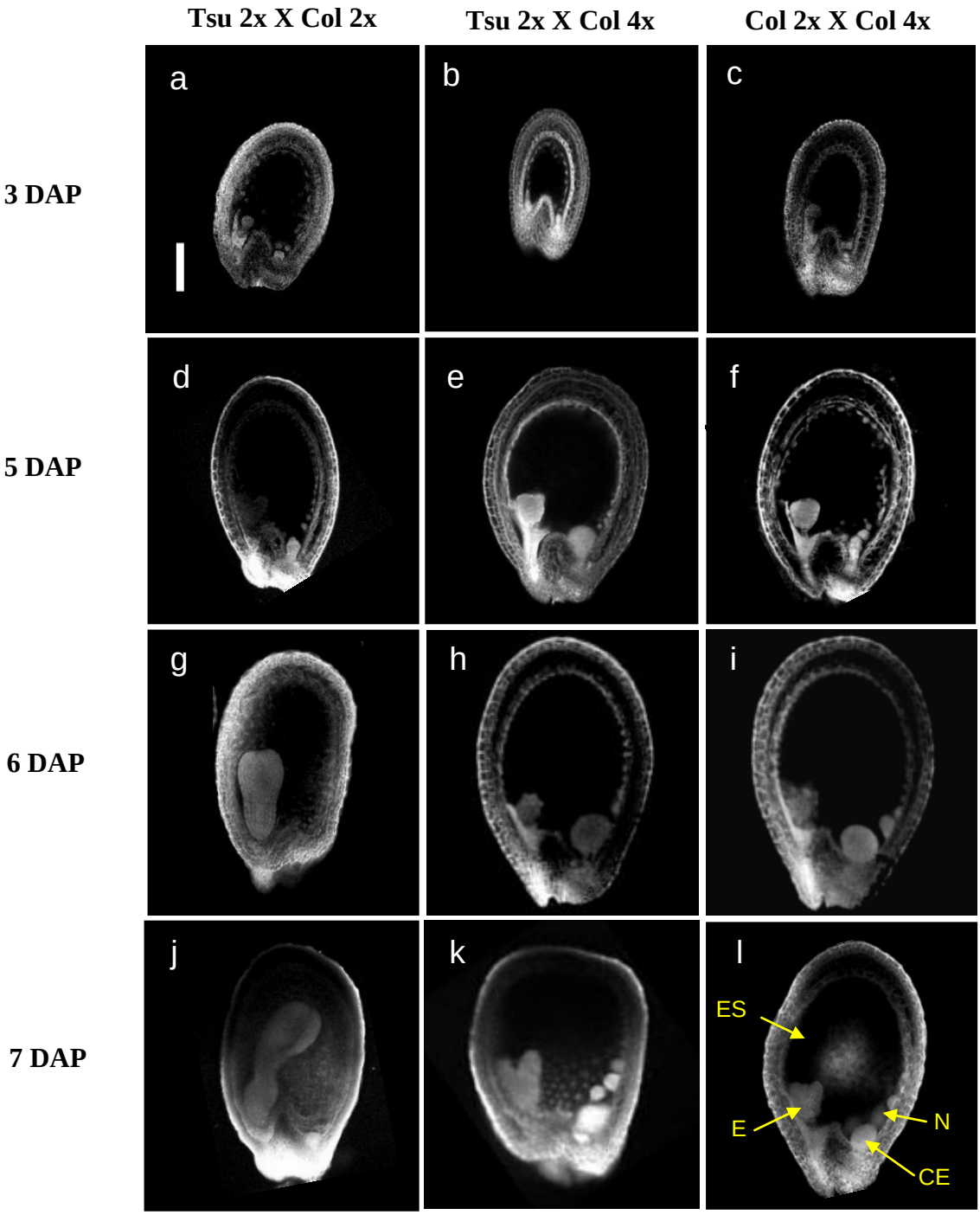

Figure 5.1: Seed development in inter-ecotype Tsu-1 and Col-0 crosses. Confocal photomicrographs of developing seeds from the inter-ecotype [Tsu 2x X Col 2x , Tsu 2x X Col 4x] and intra-ecotype [Col 2x X Col 4x] interploidy crosses are shown at 3, 5, 6, and 7 DAP. All the seeds are oriented with the embryo on the left and chalazal endosperm to the right. Scale bar = 100 µm
ES= embryo sac, E= embryo, CE= chalazal endosperm, N= nodules

5.2.1.1. Embryo sac area

Embryo sac area measurements were taken from 20-22 cleared whole-mount seeds at 3, 5, 6, and 7 DAP (Figures 5.1 and 5.2). At 3 and 5 DAP there were no significant differences in the embryo sac area of seeds produced by the three crosses. In contrast, at 6 and 7 DAP the embryo sac area of [Col 2x X Col 4x] seed was larger (116076 $\mu m^2 \pm 2392$, $P < 0.001$ and 129258 $\mu m^2 \pm 4458$, $P < 0.001$, respectively) than that of both the [Tsu 2x X Col 2x] and [Tsu 2x X Col 4x] cross. This data indicates that the Tsu-1 seed parent restricts over-proliferation of the endosperm normally induced by a Col 4x pollen parent.

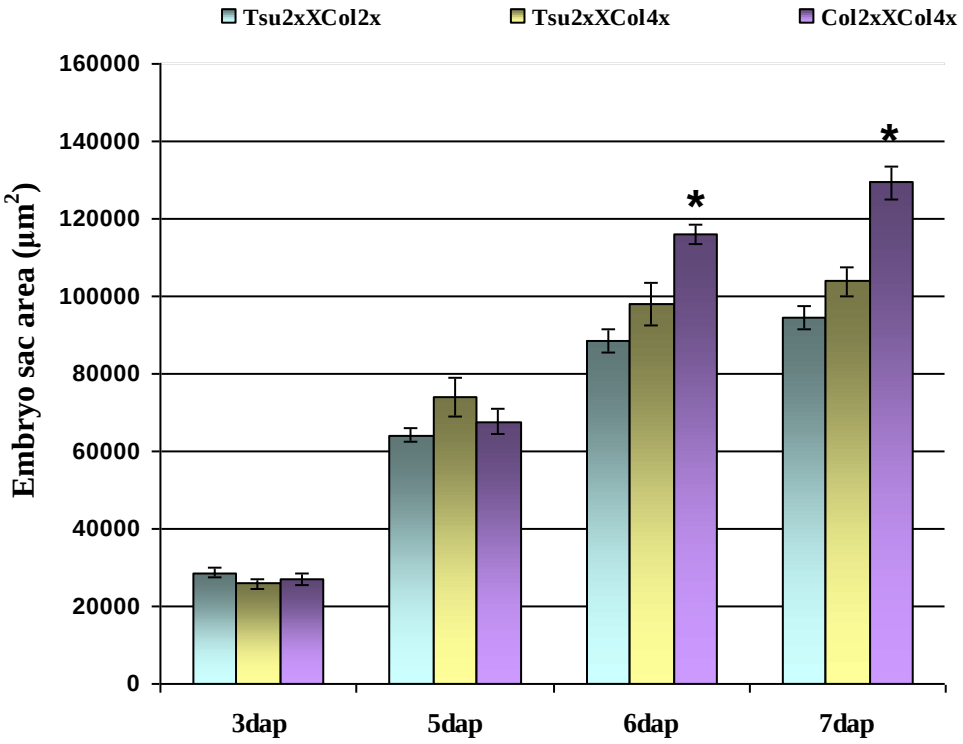

Figure 5.2: Embryo sac area associated with different interploidy crosses success. The area of embryo sac was measured using Image J at 3, 5, 6, and 7 DAP in the crosses between [Tsu 2x X Col 2x], [Tsu 2x X Col 4x], and [Col 2x X Col 4x].
***** Significant at $P < 0.05$

5.2.1.2. Chalazal endosperm and nodule size

Further studies were carried out on the size of chalazal endosperm and nodules (Figure 5.3), since these were previously observed to be larger in lethal than viable paternal excess (Scott *et al.*, 1998a). At 3 DAP, there were no significant differences between the crosses in the area of the chalazal endosperm (Figures 5.3). The chalazal endosperm area in

[Tsu 2x X Col 4x] seed was slightly larger than both [Col 2x X Col 4x] and [Tsu 2x X Col 2x] at 5 DAP. The largest differences occurred at 6 and 7 DAP when the chalazal endosperm area of [Tsu 2x X Col 4x] and [Col 2x X Col 4x] seed were considerably larger than [Tsu-1 2x X Col 2x]. This data indicates that considerable chalazal endosperm over-growth occurs in the rescuing [Tsu 2x X Col 4x] cross, but was not associated with seed failure as occurs in the [Col 2x X Col 4x] cross.

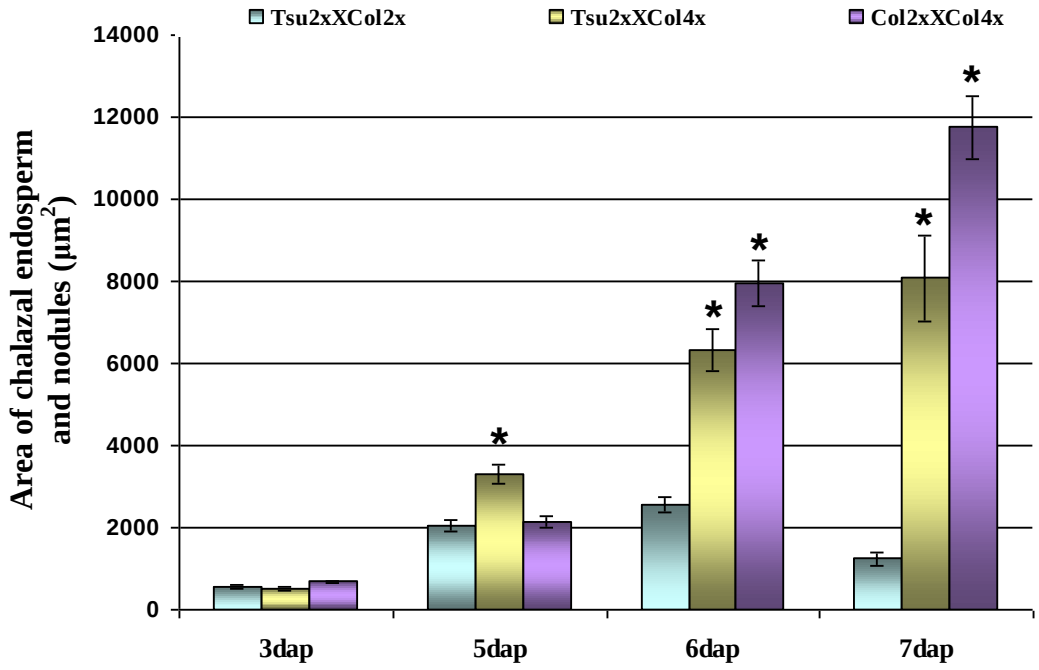

Figure 5.3: Area of chalazal endosperm and nodules
The area of chalazal endosperm and nodules in the crosses between [Tsu 2x X Col 2x], [Tsu 2x X Col 4x], and [Col 2x X Col 4x] was measured using Image J at 3, 5, 6, and 7 DAP.
***** Significant at $P < 0.05$

To further investigate the relationship between seed viability and embryo sac area, and endosperm proliferation, measurements were made of the proportion of the embryo sac area occupied by chalazal endosperm and nodules (Figure 5.4). At 6 and 7 DAP, the chalazal endosperm and nodules occupied a similar relative area (6.5-9.0%) of the embryo sac in [Tsu 2x X Col 4x] and [Col 2x X Col 4x] seed. In contrast, at the same stages the embryo sacs of [Tsu 2x X Col 4x] seed contained a small relative area (1.3-2.8%). This

data supports the findings above, that Tsu-1 rescues Col-induced lethality despite not preventing substantial endosperm over-growth.

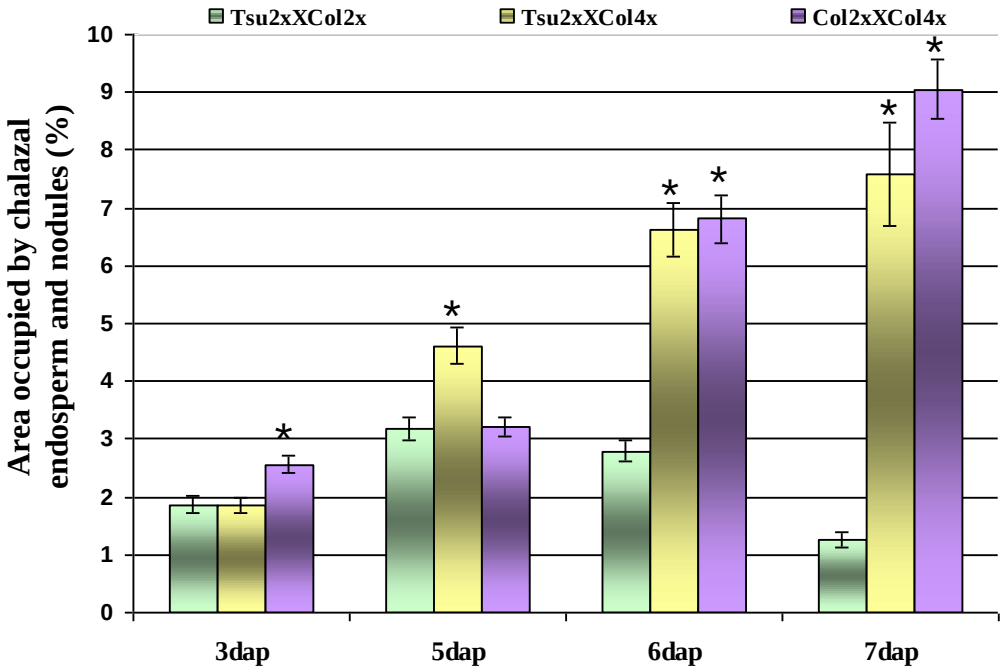

Figure 5.4: Area occupied of embryo sac occupied by chalazal endosperm and nodules

The area was measured using Image J in the crosses between [Tsu 2x X Col 2x], [Tsu 2x X Col 4x], and [Col 2x X Col 4x] at 3, 5, 6, and 7 DAP.

***** Significant at $P < 0.05$

5.2.1.3. Peripheral endosperm cellularisation

The failure of endosperm cellularisation is associated with seed failure in [Col 2x X Col 4x] crosses (Dilkes *et al.*, 2008). The frequency of endosperm cellularisation was therefore measured in seed generated by the 3 crosses at different developmental time points (Table 5.1). Consistent with previous data, [Col 2x X Col 4x] seed failed to cellularise. In contrast, [Tsu 2x X Col 2x] were fully cellularised at 6 DAP and roughly 50% of [Tsu 2x X Col 4x] seed were cellularised at 7 DAP. This shows that endosperm cellularisation occurs but is delayed in [Tsu 2x X Col 4x] seed (Images of endosperm cellularising seeds are not shown).

Table 5.1: Timing of peripheral endosperm cellularisation in inter-ecotype [Tsu2x X Col4x] balanced cross and both [Tsu2x X Col4x] and [Col2x X Col4x] interploidy crosses.
Seeds were assessed using confocal and differential interference microscopy of whole-mounts. (n= 20 seeds)

	3 DAP	5 DAP	6 DAP	7 DAP
Tsu 2x X Col 2x				
Tsu 2x X Col 4x				
Col 2x X Col 4x				

No peripheral cellularisation
Peripheral cellularisation

5.2.2. Genetic analysis of the maternal modifier behaviour of Tsu-1

5.2.2.1. Establishing a convenient and effective quantitative test for the Tsu-1 modifier behaviour (mean seed weight measure)

In order to study the genetics of the Tsu-1 maternal modifier, crosses were first conducted between Tsu-1 and Col-0 diploid seed parents and the tetraploid Col-0 as a pollen parent to confirm the earlier observations (section 4.2.3.1). The outcomes of the crosses were quantified by determining the frequency of shrivelled/plump seed and mean seed weight/silique. The mean seed weight is the total weight of the seeds in a pod divided by the number of seeds weighed. The use of mean seed weight was intended to overcome any potential problems with the subjective nature of the shrivelled seed measure. The rationale was that pods containing a high proportion of aborted seed, which have a low weight compared to plump seed, would have a low mean seed weight, and visa versa where the abortion frequency is low. Preliminary data showed that the [Tsu 2x X Col 4x] cross produced mainly plump seeds (96.7 % ± 0.59; Student's t-test; $P < 0.001$) compared to the [Col 2x X Col 4x] control cross (Figure 5.6). The mean seed weight for the [Tsu 2x X Col 4x] cross was ~ 39µg (Figure 5.7). This supports the idea that mean seed weight is positively correlated with a high frequency of plump seed, or a low frequency of shrivelled seed. In contrast, the [Col 2x X Col 4x] cross produced a high frequency of shrivelled seed (87.5 % ± 1.27, Student's t-test; $P < 0.001$) compared to almost complete absence of shrivelled seed in the [Tsu-1 2x X Col 2x] cross (Figure 5.8a). The mean weight of [Col 2x

X Col 4x] seeds was ~ 28µg. Again the mean seed weight and the frequency of plump/shrivelled seeds are correlated in the way excepted (Figure 5.7 and Figure 5.8a-c). The above data confirms that Tsu-1 acts as a strong maternal modifier of Col-induced seed killing.

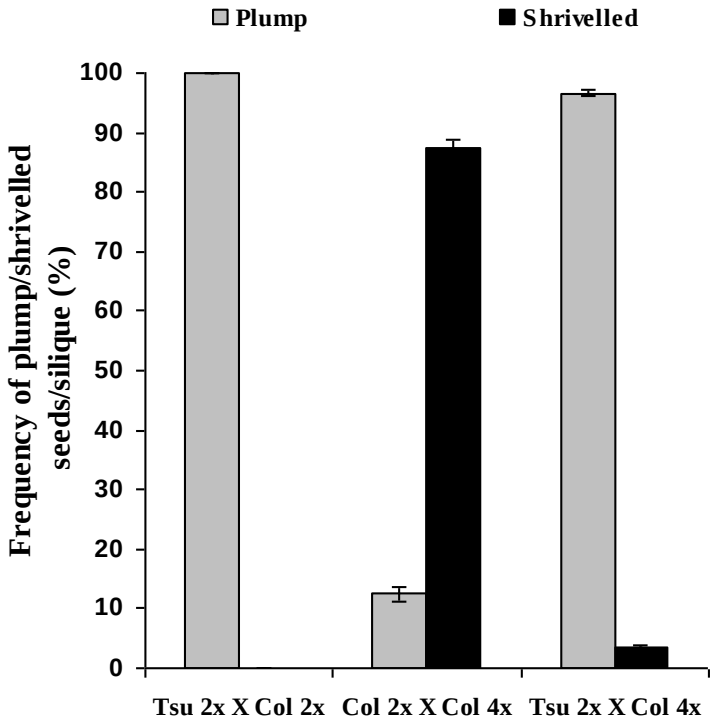

Figure 5.6: Genetic background determines the level of seed survival in inter-ecotype [Tsu2x X Col2x , Tsu2x X Col4x] and intra-ecotype [Col2x X Col4x] interploidy crosses. The frequency of the plump and shrivelled seeds in the progeny of these crosses (y-axis) was determined and the mean for each cross is given along with the standard error. (n= 6 siliques) Significant at $p < 0.001$

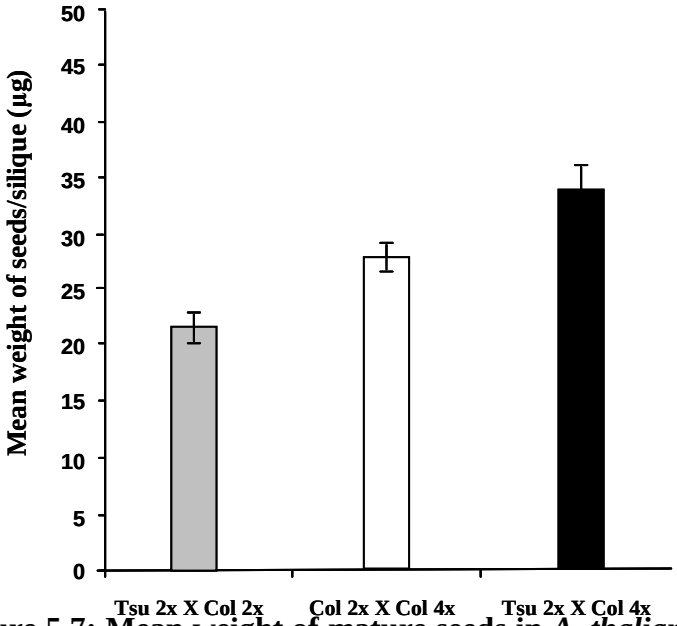

Figure 5.7: Mean weight of mature seeds in *A. thaliana* [Tsu 2x X Col 2x], [Col 2x X Col 4x], and [Tsu 2x X Col 4x] interploidy crosses. The mean weight of seeds in the progeny of these crosses (y-axis) was determined and is given along with the standard error for each cross. (n= 6 siliques)
Significant at $P < 0.001$

Figure 5.8: Seeds produced following inta- and inter-ecotype interploidy crosses showing the variation in abnormal development in *A. thaliana* Tsu-1 and Col-0 ecotypes in order to study the resistance/ sensitivity to the killing behaviour of Col-0.
(a) Tsu 2x X Col 2x (b) Col 2x X Col 4x (c) Tsu 2x X Col 4x. Scale bar = 1mm

5.2.2.2. Genetic analysis of the F_1 generation – Is the Tsu-1 modifier dominant or recessive?

As a first step to understanding the genetic basis of the Tsu-1 modifier, experiments were conducted to establish whether the modifier trait was dominant or recessive. The genetic analysis of the Tsu-1 modifier was carried out using a recurrent backcrossing method (Figure 5.9), to obtain a $BC3F_3$ generation.

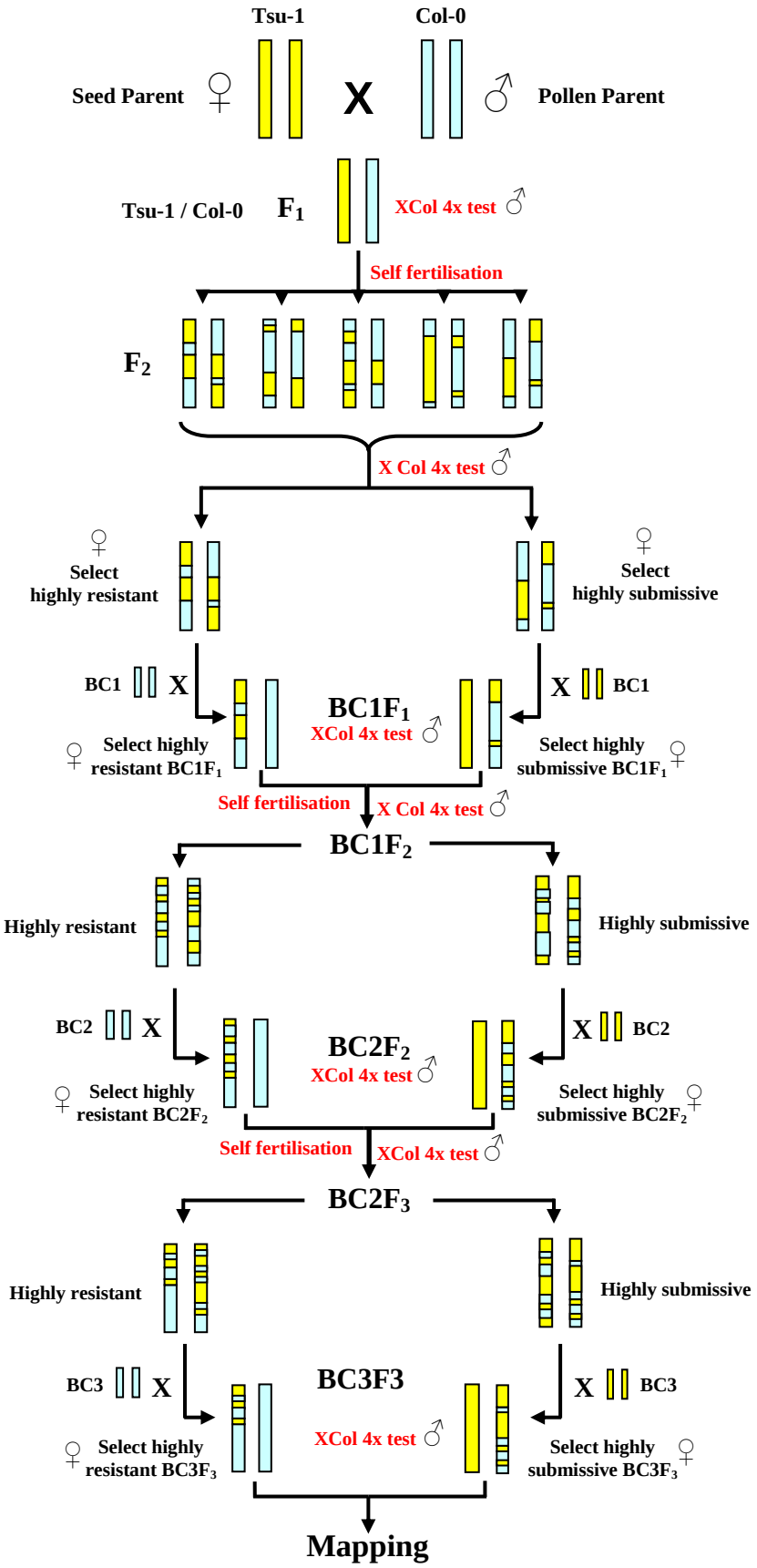

Figure 5.9: Diagram of the main types of mapping populations developed using two homozygous diploid parents, Tsu-1 (seed parent) and Col-0 (pollen parent). The process of mapping involved three recurrent backcrossings and at each step the selection of individuals displaying either high (submissive) or low (resistant) levels of abnormal seed production in crosses to Col 4x.

F_1 plants were made by crossing diploid Tsu-1 as seed parent with a diploid Col-0 as pollen parent, as shown before in Figure 5.9. The heterozygosity of the resulting F_1 progeny was confirmed using co-dominant CAPs markers (Figure 5.10)

The ability of the F_1 plants to resist Col-killing was tested using a Col 4x pollen parent and the outcome quantified using the mean seed weight measure (Figure 5.11). The preliminary data showed that all F_1 lines crossed with Col 4x produced seeds that were significantly different in weight to [Col 2x X Col 4x], (Student's t-test, [Col 2x X Col 4x] vs plant 1-15, $P \leq 0.001$). In contrast, F_1 plants 2, 6, 8, 9, 10, 11, 12, 13, 14, and 15 showed no significant difference in their weight to [Tsu 2x X Col 4x]. Consequently, there was significance in the mean weight of seeds for F_1 plants 1, 3, 4, 5, and 7 compared to the [Tsu 2x X Col 4x] (Student's t-test, [Tsu 2x X Col 4x]; vs plant 1, P= 0.014; vs plant 3, P= 0.021; vs plant 4, P= 0.037; vs plant 5, P= 0.007; vs plant 7, P= 0.029). The mean seed weight of F_1 plants crossed to Col 4x ranged between ~ 25-48µg compared to ~18µg for the [Col 2x X Col 4x] control cross, while it was ~35µg for [Tsu 2x X Col 4x] cross indicating that F_1 plants are effective rescuers. Images of different [F_1 X Col 4x] seeds are shown in Figure 5.12. From the previous analysis of the F_1 populations, we conclude that about 8 different plants behaved like Tsu-1 while none of these F_1 plants showed Col-0 behaviour.

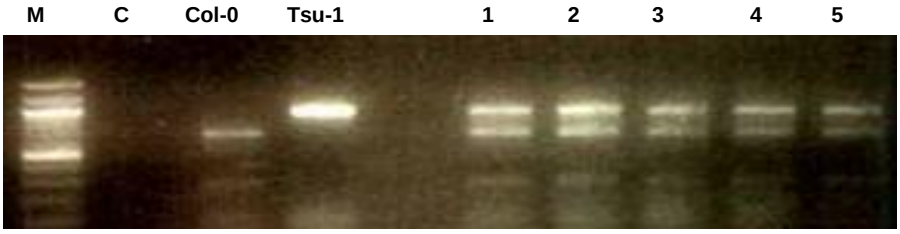

Figure 5.10: Segregation patterns for co-dominant markers in five different F_1 [Tsu 2x X Col 2x] plants. The restriction analysis of the F_1 plants confirmed that all F_1 were heterozygous. M= DNA ladder (100bp); C= Control (H_2O), 1, 2, 3, 4, and 5 = different F_1 plants.

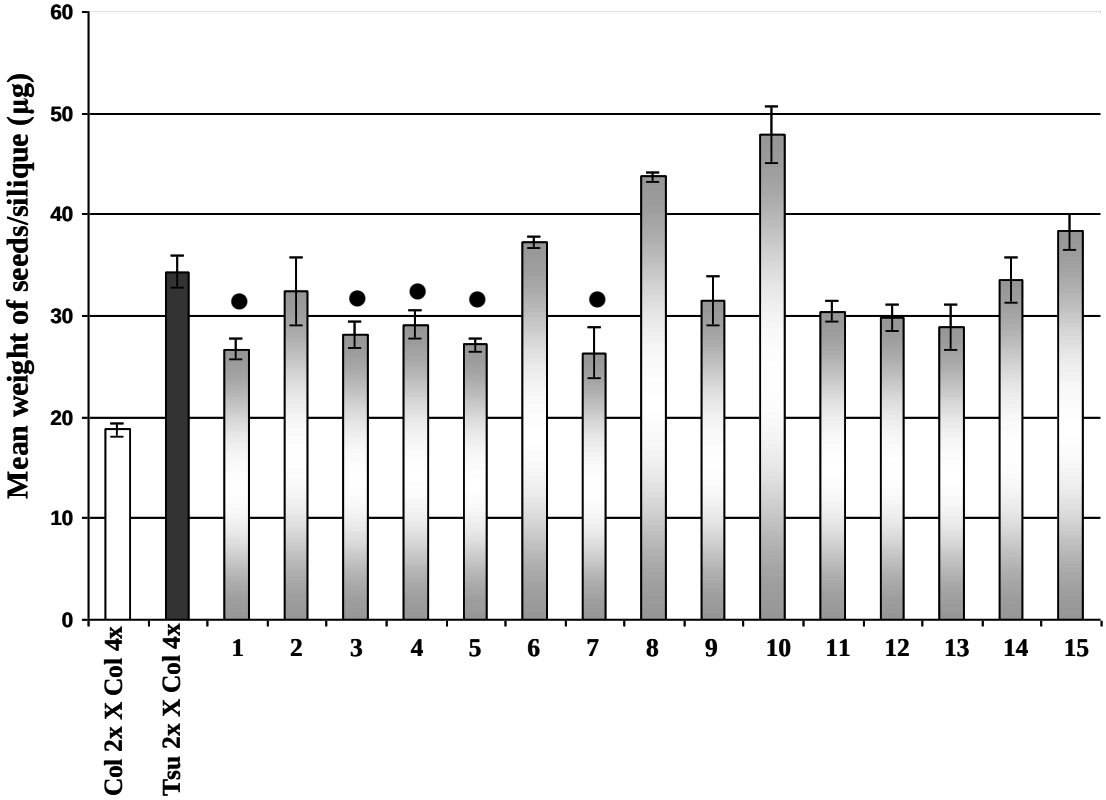

Figure 5.11: F_1 Tsu-1/Col-0 plants rescue Col-killing.
The mean weight of F_1 seeds were measured and compared in 15 F_1 progenies to both control crosses [Tsu 2x X Col 4x] and [Col 2x X Col 4x] to identify individuals highly resistant or highly submissive to the killing behaviour of Col-0.
All plants are significant at $P < 0.05$ with the [Col 2x X Col 4x] interploidy cross.
● Significant at $P < 0.05$ with [Tsu 2x X Col 4x] interploidy cross.

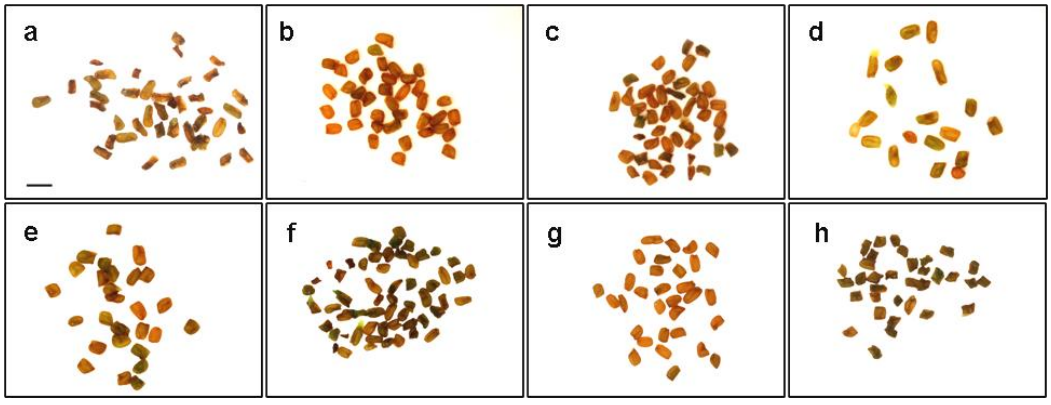

Figure 5.12: : Phenotypes of seeds produced in crosses between F_1 Tsu-1/Col-0 plants and 4x Col. Photomicrographs of the mature seed phenotypes developed from the interploidy cross between diploid F_1 (Tsu2xXCol2x) hybrids and Col4x in order to identify both highly resistant and highly submissive plants to the Col-0 killing effect. (a) Col 2x X Col 4x (b) Tsu 2x X Col 4x (c, d, e, f, g, and h) Seeds from different F_1 [Tsu 2x X Col 2x] X Col 4x populations. Scale bar = 1mm

To confirm that F_1 plants are effective rescuers of Col-killing, we performed an analysis for the average mean weight of seeds for [F_1 X Col 4x] (Figure 5.13). The average seed weight/silique for [F_1 X Col 4x] plants was (32.8 µg ± 1.66) compared to (34.3 µg ± 1.54) for [Tsu 2x X Col 4x], (Figure 5.13). Statistical analysis using Mann-Whitney *U*-test found no significant difference between these values, indicating that the Tsu-1/Col-0 F_1 hybrid resists Col-killing almost as effectively as the Tsu-1 parent. In contrast, the average mean seed weight was significantly different to [Col 2x X Col 4x] which had a value of (18.8 µg ± 0.61, Mann-Whitney *U*-test; P≤ 0.001). The seed weight analysis suggests that one or more Tsu-1 modifier loci are functionally dominant.

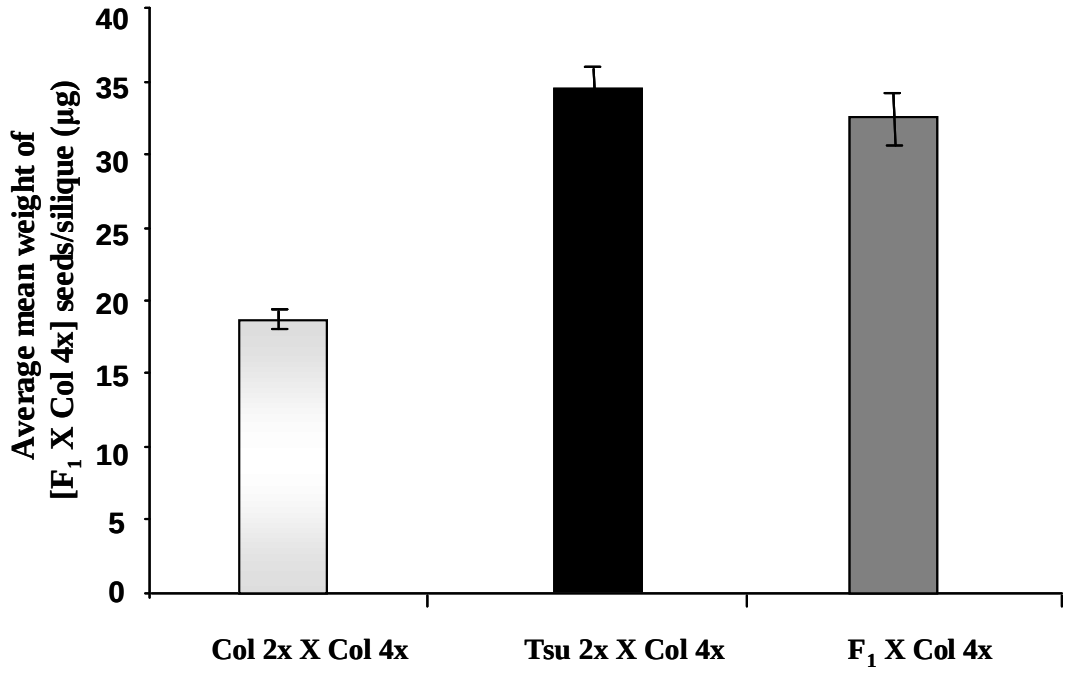

Figure 5.13: Average mean weight of mature seeds following interploidy [F_1XCol4x] crosses compared to control crosses [Tsu 2x X Col 4x] and [Col 2x X Col 4x]. n= 3-5 siliques.
Significant at P ≤ 0.001 with [Col 2x X Col 4x] interploidy cross.
Non-significant at P= 0.257 with [Tsu 2x X Col 4x] interploidy cross.

5.2.2.3. Analysis of the F_2 generation

The aim of this experiment is to study the genetics of the Tsu-1 modifier by selecting the most 4x Col-0 resistant and submissive F_2 plants on which to perform the recurrent backcrossing strategy. F_2 plants were produced by selfing F_1 hybrids from the cross between the two parents Tsu-1 and Col-0 as shown before in Figure 5.9. Approximately 70 F_2 plants were grown from F_2 seeds and crossed as seed parent to Col 4x. The resulting siliques were harvested at maturity and the percentage of plump and shrivelled seeds recorded. There was near continuous variation in plump/shrivelled seed frequency across the population of F_2 plants, including plants with seed phenotypes close to each parent (Figures 5.14 and 5.17). Individuals in these extreme groups were classified as either resistant (shrivelled seed frequency < 10%) or submissive (shrivelled seed frequency >90%).

To give greater confidence in selecting resistant and submissive individuals for further backcrossing from the F_2 population, the mean seed weight of the [F_2 X Col 4x] seeds was also determined and compared to [Tsu 2x X Col 4x] and [Col 2x X Col 4x] as controls. Between 3 and 5 siliques per plant were analysed, and the data used to select the 5 most resistant and the 3 most submissive plants (Figures 5.15 and 5.16). The mean weight of [F_2 X Col 4x] seed for the 5 resistant plants showed no significant difference to control [Tsu 2x X Col 4x] seed (34.0 µg ± 1.30 vs 29.3 µg ± 0.35; ANOVA followed by Tukey's multiple comparisons, P= 0.060) (Figure 5.15). Figure 5.17c,d shows mature seed from two resistant lines; the seed was mostly plump and resembled [Tsu-1 X Col 4x] seed (Figure 5.17b). This data indicated that these particular F_2 plants had the same levels of resistance to 4x Col pollen as the Tsu-1 parent, presumably due to inheriting the maternal modifier gene or genes. The 3 submissive plants each had a lower mean seed weight than [Col 2x X Col 4x] seed (7.9 µg ± 0.95 vs 18.7 µg ± 1.87; Student's t-test, P= 0.007) (Figure 5.16). Figure 5.17g,h shows mature seed from two submissive lines; the seed was mostly shrivelled and resembled [Col 2x X Col 4x] seed (Figure 5.17a). This data indicated that these particular F_2 plants were highly submissive to 4x Col-0 pollen, presumably due to not inheriting the Tsu-1 maternal modifier gene or genes.

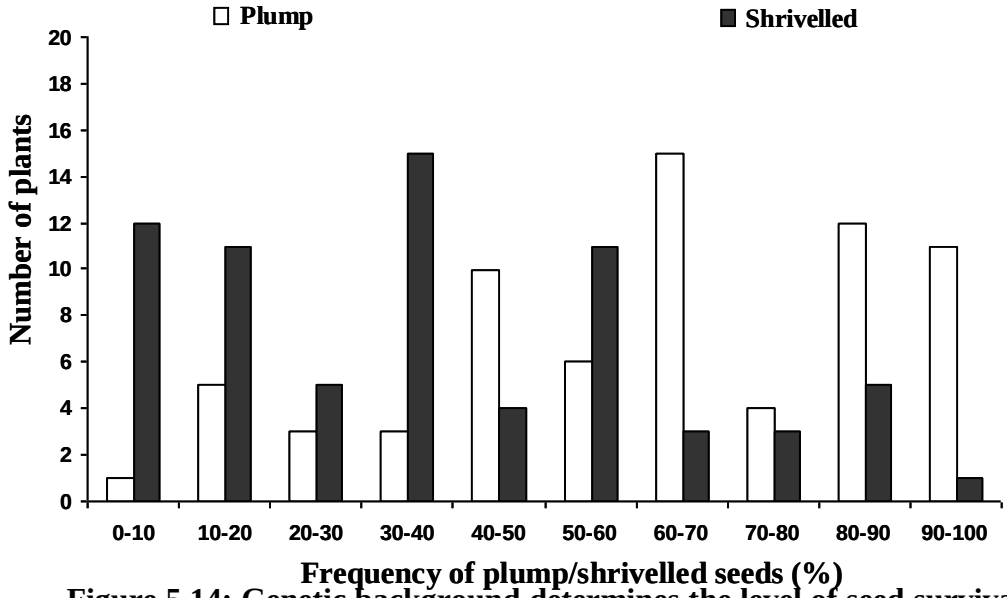

Figure 5.14: Genetic background determines the level of seed survival in F_2 hybrids. The frequency of plump and shrivelled seeds was quantified individually in total 70 seed parents F_2 plants derived from selfed F_1 hybrid progenies and crossed with Col 4x pollen parent. ($n \leq 6$ siliques)

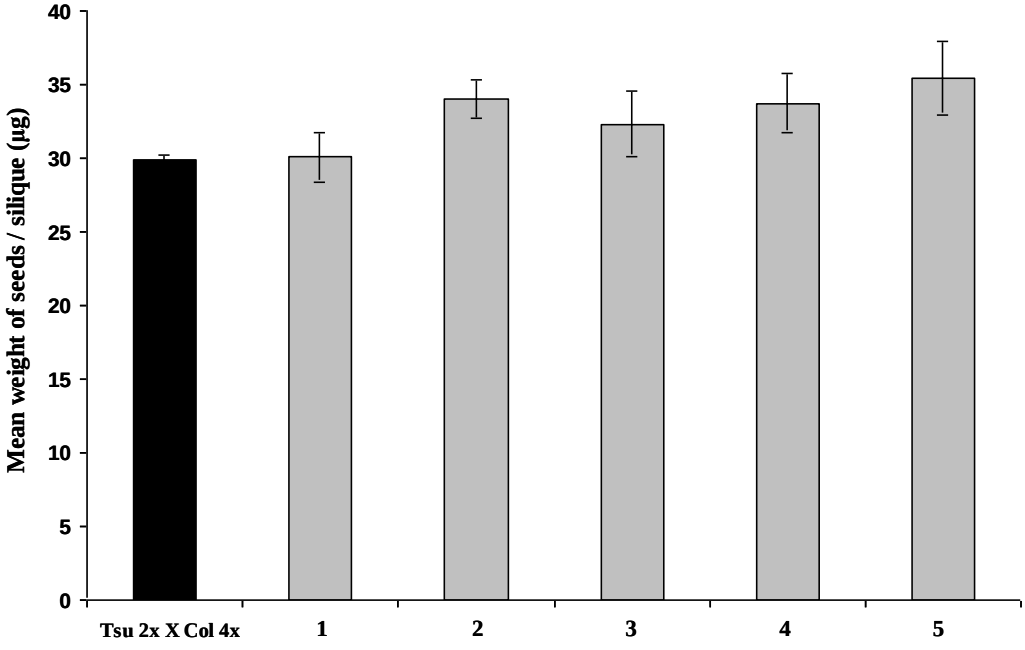

Figure 5.15: Mean seed weight analysis of selected 4x Col-0 resistant Tsu-1/Col-0 F_2 plants. The mean weight of mature seeds was quantified in 5 highly resistant [F_2 X Col 4x] plants generated from the interploidy cross between diploid Tsu-1 and Col-0. ($n \leq 5$ siliques)
Non-significant with [Tsu 2x X Col 4x]

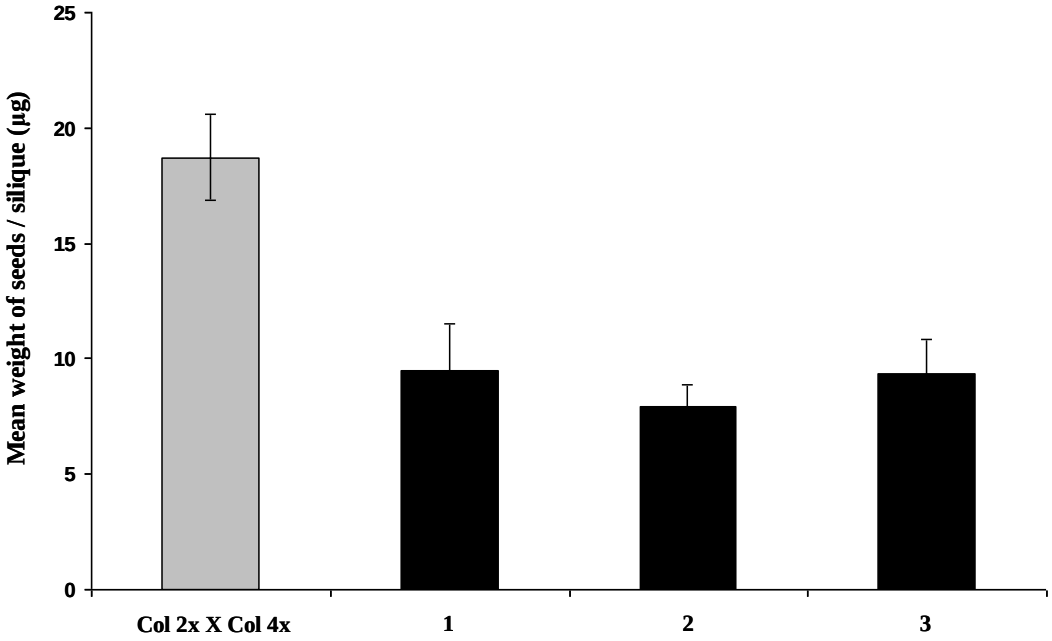

Figure 5.16: Mean seed weight analysis of selected 4x Col-0 submissive Tsu-1/Col-0 F_2 plants. The mean weight of mature seeds was quantified in 5 highly submissive [F_2 X Col 4x] plants generated from the interploidy cross between diploid Tsu-1 and Col-0. ($n \leq 5$ siliques)
Significant with [Col 2x X Col 4x].

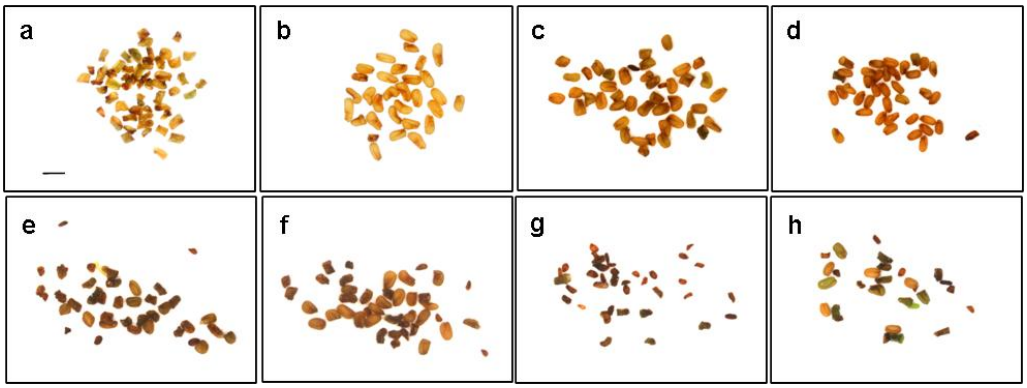

Figure 5.17: Phenotypes of seed produced by selected resistant and submissive Tsu-1/Col-0 F_2 plants following a cross to 4x Col-0 pollen.
(a) Col 2x X Col 4x (b) Tsu 2x X Col 4x. (c,d) seed from resistant plants. (e,f) seed from intermediate plants. (g,h) seed from submissive plants. Scale bar = 1mm.

5.2.3. Mapping the maternal modifier trait

The next step in mapping the Tsu-1 modifier gene was to perform a series of recurrent backcrosses starting from the selected highly resistant and submissive F_2 plants described in the previous section. The objective was to generate two $BC3F_3$ mapping populations: a highly resistant line produced by recurrent backcrossing to the submissive parent (Col-0) and the other a highly submissive line produced by recurrent backcrossing to the resistant parent (Tsu-1) (Figure 5.9). These plants would have the Tsu-1 and Col-0 alleles of the modifier locus/loci in a genetic background enriched for DNA of the parent with the opposite modifier behaviour. The DNA of a resistant plant would consist of a small amount of Tsu-1 DNA carrying the Tsu-1 modifier locus, and a relatively large amount Col DNA, and *visa versa* for a submissive plant.

The selection of resistant or submissive plants at each round of backcrossing was performed on F_2 plants and was based on their performance in a test cross to 4x Col-0. The principal performance criteria were, 1) mean seed weight - the highest value in the case of backcrosses to Col-0, and the lowest value for backcrosses to Tsu-1 BC; 2) close morphological similarity of the test seed to the control cross seed [Tsu 2x X Col 4x] or [Col 2x X Col 4x]. F_3 seed was collected from plants satisfying these criteria and used to produce plants for the next round of backcrossing.

5.2.3.1. Identification of resistant BC1(Col-0)F_1 and submissive BC1(Tsu-1)F_1 plants

The 5 highly resistant Tsu-1/Col-0 F_2 plants were backcrossed to the Col-0 2x (submissive donor) to generate BC1(Col-0)F_1 seed (Figure 5.9). Subsequently, 10 seeds from each BC1(Col-0)F_1 plant were sown on soil; a total of 39 plants were produced (11 seed failed to germinate). These plants were used as seed parents in crosses with 4x Col-0 pollen and the mean weight of the resulting seeds determined (Table 5.2). There was considerable variation for mean seed weight across the population of plants, with the majority of values below that of the resistant parent (Tsu-1). One plant (number 12) produced mostly large plump seed with a mean weight of 40.7 μg ± 2.0, compared to the control value for [Tsu 2x x Col 4x] seed (29.9 ± 0.4) (Figure 5.18a , b). This plant met the selection criteria and was entered into the next round of back crossing.

The 3 highly submissive Tsu-1/Col-0 F_2 plants were backcrossed to the Tsu-1 (resistant donor) to generate BC1(Tsu-1)F_1 seed (Figure 5.9). Again, 10 seed from each

BC1(Tsu-1)F_1 plant were sown on soil; a total of 23 plants were produced (7 seed failed to germinate). These plants were used as seed parents in crosses with 4x Col-0 pollen and the mean weight of the resulting seeds determined (Table 5.3). The data revealed variation across the plants, but with only a minority of plants that with a mean seed weight not significantly different to the [Col 2x X Col 4x] control ($P > 0.05$), indicating a low frequency inheritance of the modifier trait from the Col-0 parent or dominance of the Tsu-1 modifier. The lowest mean seed weight was 14.9 µg ± 1.1 (plant 20) which was significantly lower than the [Col 2x X Col 4x] control cross (23.9 µg ± 1.5; Student's t-test, $P= 0.003$) (Figure 5.18c, d). This plant met the selection criteria and was entered into the next round of backcrossing.

Table 5.2: Identification of Col 4x resistant BC1(Col-0)F_1 plants. BC1(Col-0)F_1 plants were crossed with Col 4x pollen and the mean seed weight of the resulting seeds determined. P values are for a comparison between [BC1(Col-0)F_1 X Col 4x] and the control cross, [Tsu 2x X Col 4x]**.** The plant selected for entry into the next round of back-crossing is highlighted in blue.

Cross	Number of Siliques	Mean seed weight (µg) ± se	P value
[Tsu 2x X Col 4x]	3	29.9 ± 0.4	
BC1(Col-0)F1 X Col4x			
1	6	22.3 ± 1.4	0.003
2	6	29.1 ± 0.9	0.455
3	4	11.3 ± 0.3	0.001
4	5	32.2 ± 0.9	0.061
5	5	11.0 ± 1.0	0.001
6	5	23.1 ± 2.0	0.031
7	6	25.1 ± 2.2	0.077
8	6	30.4 ± 1.2	0.736
9	6	28.1 ± 0.8	0.094
10	6	26.3 ± 1.7	0.102
11	5	25.7 ± 2.5	0.171
12	5	40.7 ± 2.0	0.006
13	3	30.4 ± 1.8	0.794
14	4	28.0 ± 1.2	0.216
15	4	27.9 ± 3.3	0.576
16	8	26.3 ± 2.1	0.128
17	3	8.2 ± 1.1	0.003
18	3	8.0 ± 2.5	0.013
19	3	35.5 ± 1.4	0.009
20	6	17.8 ± 2.6	0.005
21	6	24.7 ± 0.9	0.001
22	6	28.9 ± 0.4	0.116
23	6	16.8 ± 2.9	0.006
24	6	25.9 ± 1.1	0.021
25	6	27.3 ± 1.2	0.095
26	6	30.4 ± 1.7	0.787
27	5	24.5 ± 1.6	0.032
28	6	20.9 ± 2.1	0.008
29	6	20.6 ± 2.0	0.005
30	6	28.3 ± 1.7	0.376
31	4	15.1 ± 1.2	0.001
32	4	21.9 ± 0.9	0.004
33	4	27.3 ± 2.4	0.348
34	4	19.4 ± 4.6	0.109
35	5	31.1 ± 3.8	0.069
36	3	10.0 ± 2.3	0.014
37	3	12.2 ± 1.9	0.011
38	3	10.4 ± 2.5	0.016
39	3	6.4 ± 0.6	0.001

Table 5.3: Identification of Col 4x submissive BC1(Tsu-1)F_1 plants. BC1(Tsu-1)F_1 plants were crossed with Col 4x pollen and the mean seed weight of the resulting seeds determined. P values are for a comparison between [BC1(Tsu-1)F_1 X Col 4x] and the control cross, [Tsu 2x X Col 4x]**.** The plant selected for entry into the next round of back-crossing is highlighted in yellow.

Cross	Number of Siliques	Mean seed weight (µg) ± se	P value
[Col 2x X Col 4x]	3	23.9 ± 1.5	
BC1(Tsu-1)F1 X Col 4x			
1	4	50.5 ± 3.1	0.001
2	4	34.6 ± 3.8	0.069
3	4	36.4 ± 4.9	0.085
4	4	48.9 ± 2.0	0.001
5	4	45.1 ± 2.6	0.001
6	4	25.9 ± 3.5	0.653
7	4	47.5 ± 1.3	0.001
8	4	16.0 ± 1.4	0.014
9	4	47.6 ± 2.3	0.001
10	4	43.1 ± 4.4	0.016
11	4	42.0 ± 5.8	0.048
12	5	42.2 ± 2.6	0.003
13	4	36.8 ± 0.7	0.001
14	4	43.4 ± 0.8	0.001
15	5	36.4 ± 2.4	0.009
16	4	31.8 ± 3.8	0.148
17	3	31.2 ± 3.2	0.105
18	4	40.1 ± 4.3	0.026
19	4	36.2 ± 2.3	0.009
20	5	14.9 ± 1.1	0.003
21	3	45.3 ± 2.5	0.002
22	3	46.4 ± 3.3	0.004
23	4	26.4 ± 1.4	0.012

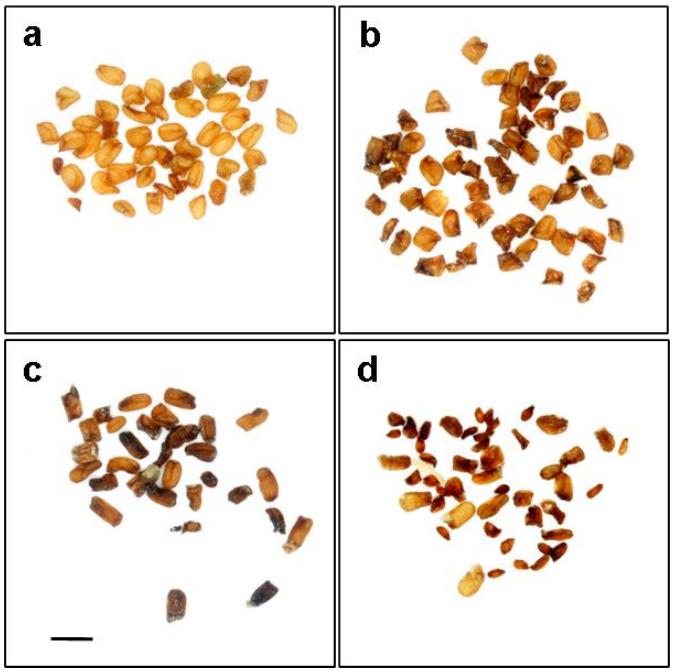

Figure 5.18: Seeds produced in [BC1F$_1$XCol4x] test crosses showing variation in seed development. (a) [Tsu 2x X Col 4x] control seed (b) seed from a highly Col-0 resistant plant (c) [Col 2x X Col 4x] control seed (d) seed from Col-0 highly submissive plant. Scale bar = 1mm

5.2.3.2. Identification of resistant BC1(Col-0)F$_2$ and submissive BC1(Tsu-1)F$_2$ plants

To produce BC1F$_2$ populations, the selected highly resistant and highly submissive BC1F$_1$ plants (numbers 12 and 20 respectively) were allowed to self-fertilise (Figure 5.9). A total of 15 BC1(Col-0)F$_2$ plants were produced from seed from the highly resistant plant (number 12; Table 5.2), and the mean seed weight determined for each following a cross to 4x Col-0 (Table 5.4). The majority of plants (10 from 15) had mean seed weights that were not significantly different to the [Tsu 2x X Col 4x] control ($P > 0.05$), indicating high frequency inheritance and/or dominance of the modifier trait from the Tsu-1 parent. An example of seed from a highly resistant BC1(Col-0)F$_2$ plant (number 9 in Table 5.4) is shown in Figure 5.19 b. This plant met the selection criteria and was entered into the next round of backcrossing.

A total of 10 BC1(Tsu-1)F$_2$ plants were grown from seed of the selected highly submissive BC1(Tsu-1)F$_1$ plant (number 20: Table 5.3) and crossed with Col 4x pollen to determine the mean weight of seeds (Table 5.5). Only a minority of plants (2 from 10) had mean seed weights that were not significantly different to the [Col 2x X Col 4x] control (P

> 0.05), indicating low frequency inheritance of the modifier trait from the Col-0 parent or dominance of the Tsu-1 modifier inherited from the Tsu-1 recurrent parent. An example of seed from a highly submissive BC1(Tsu-1)F_2 plant (number 5: Table 5.5) is shown in Figure 5.19d. This plant met the selection criteria and was entered into the next round of backcrossing.

Table 5.4: Identification of Col 4x resistant BC1(Col-0)F_2 plants. BC1(Col-0)F_2 plants were crossed with Col 4x pollen and the mean seed weight of the resulting seeds determined. P values are for a comparison between [BC1(Col-0)F_2X Col 4x] and the control cross, [Tsu 2x X Col 4x]**.** The plant selected for entry into the next round of back-crossing is highlighted in blue

Cross	Number of Siliques	Mean seed weight (µg) ± se	P value
[Tsu 2x X Col 4x]	3	28.49 ± 0.24	
BC1(Col-0)F2 X Col4x			
1	4	24.5 ± 1.0	0.022
2	4	21.8 ± 1.1	0.004
3	4	29.7 ± 1.0	0.365
4	3	28.3 ± 0.4	0.687
5	4	26.9 ± 0.8	0.123
6	4	28.0 ± 1.2	0.759
7	4	27.7 ± 0.8	0.382
8	3	30.7 ± 0.5	0.013
9	4	31.4 ± 1.1	0.072
10	4	31.0 ± 1.5	0.176
11	4	30.7 ± 0.5	0.014
12	3	25.2 ± 0.3	0.001
13	4	29.6 ± 0.5	0.145
14	4	31.0 ± 0.8	0.055
15	4	29.7 ± 0.6	0.171

Table 5.5: Identification of Col 4x submissive BC1(Tsu-1)F_2 plants. BC1(Tsu-1)F_2 plants were crossed with Col 4x pollen and the mean seed weight of the resulting seeds determined. P values are for a comparison between [BC1(Tsu-1)F_1 X Col 4x] and the control cross, [Col 2x X Col 4x]**.** The plant selected for entry into the next round of back-crossing is highlighted in yellow.

Cross	Number of Siliques	Mean seed weight (µg) ± se	P value
[Col 2x X Col 4x]	3	16.6 ± 1.6	
BC1(Tsu-1)F2 X Col 4x			
1	4	25.4 ± 2.8	0.057
2	3	36.9 ± 1.2	0.001
3	4	30.3 ± 2.1	0.005
4	4	20.0 ± 3.1	0.394
5	4	8.2 ± 1.3	0.006
6	4	32.3 ± 1.8	0.003
7	4	30.0 ± 1.6	0.004
8	4	32.3 ± 3.6	0.016
9	3	31.9 ± 1.6	0.003
10	4	15.9 ± 2.7	0.848

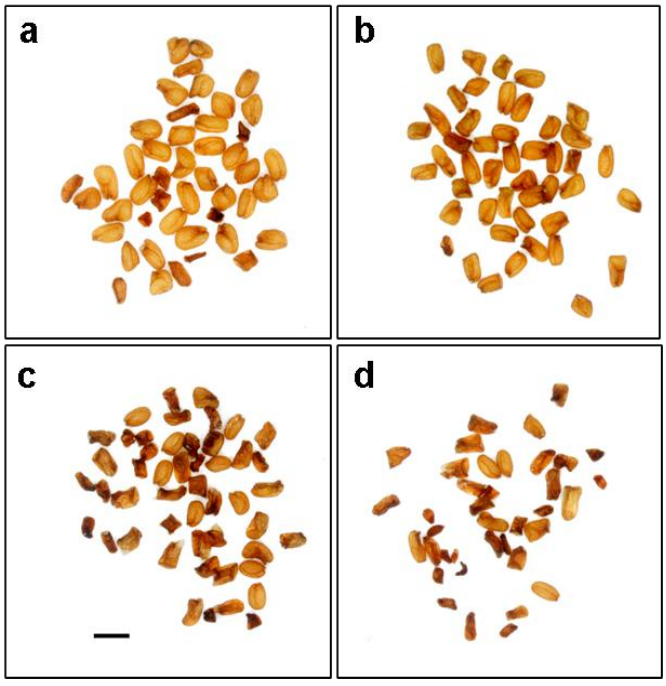

Figure 5.19: Seeds produced in [BC1F_2XCol4x] test crosses showing variation in seed development. (a) [Tsu 2x X Col 4x] control seed (b) seed from a highly Col-0 resistant plant (c) [Col 2x X Col 4x] control seed (d) seed from Col-0 highly submissive plant. Scale bar = 1mm

5.2.3.3. Identification of resistant BC2(Col-0)F_2 and submissive BC2(Tsu-1)F_2 plants

The process of generating resistant and submissive BC3F_3 plants was continued by backcrossing the BC1F_2 plants described in the previous section to the appropriate recurrent parent – Col-0 for resistant plant BC1(Col-0)F_2 number 9 and Tsu-1 for submissive plant BC1(Tsu-1)F_2 number 5 (Figure 5.9).

A total of 32 BC2(Col-0)F_2 plants were produced from seed from the highly resistant plant BC1(Col-0)F_2 (number 9: Table 5.4), and the mean seed weight determined for each following a cross to 4x Col-0 (Table 5.6). The majority of plants (26 from 32) had mean seed weights that were not significantly different to the [Tsu 2x X Col 4x] control ($P > 0.05$), indicating high frequency inheritance and/or dominance of the modifier trait from the Tsu-1 parent. An example of seed from a highly resistant BC2(Col-0)F_2 plant (number 26: Table 5.6) is shown in Figure 5.20b. This plant had an exceptionally high mean seed weight and good seed morphology, and was the selected for entry into the next round of backcrossing.

A total of 34 BC2(Tsu-1)F_2 plants were grown from seed of the selected highly submissive BC1(Tsu-1)F_2 plant (number 5: Table 5.5) and crossed with Col 4x pollen to determine the mean weight of seeds (Table 5.7). Only a minority of plants (7 from 32) had mean seed weights that were not significantly different to the [Col 2x X Col 4x] control ($P > 0.05$), indicating low frequency inheritance of the modifier trait from the Col-0 parent or dominance of the Tsu-1 modifier. An example of seed from a highly submissive BC2(Tsu-1)F_2 plant (number 24: Table 5.7) is shown in Figure 5.20d. This plant met the selection criteria and was entered into the next round of backcrossing.

Table 5.6: Identification of Col 4x resistant BC2(Col-0)F_2 plants. BC2(Col-0)F_2 plants were crossed with Col 4x pollen and the mean seed weight of the resulting seeds determined. P values are for a comparison between [BC2(Col-0)F_2X Col 4x] and the control cross, [Tsu 2x X Col 4x]**.** The plant selected for entry into the next round of back-crossing is highlighted in blue.

Cross	Number of Siliques	Mean seed weight (µg) ± se	P value
[Tsu 2x X Col 4x]	4	30.3 ± 1.4	
BC2(Col-0)F2 X Col4x			
1	4	33.4 ± 0.5	0.051
2	4	37.3 ± 1.9	0.042
3	3	33.6 ± 1.3	0.155
4	4	38.4 ± 1.0	0.005
5	4	27.6 ± 0.8	0.134
6	4	29.2 ± 3.0	0.758
7	4	29.8 ± 2.4	0.856
8	4	33.4 ± 0.4	0.061
9	4	22.2 ± 1.8	0.021
10	4	26.1 ± 2.0	0.175
11	4	32.3 ± 0.6	0.195
12	3	28.5 ± 1.1	0.355
13	3	29.1 ± 0.1	0.423
14	3	32.1 ± 0.5	0.282
15	4	30.2 ± 2.9	0.963
16	4	30.2 ± 0.6	0.931
17	4	33.7 ± 0.6	0.053
18	4	33.2 ± 0.5	0.123
19	3	28.2 ± 0.3	0.217
20	3	30.5 ± 1.2	0.931
21	3	31.3 ± 1.2	0.607
22	4	37.9 ± 1.4	0.014
23	4	34.2 ± 1.7	0.157
24	4	30.5 ± 0.9	0.895
25	4	26.3 ± 0.8	0.065
26	4	38.6 ± 1.5	0.011
27	4	26.9 ± 1.7	0.198
28	4	32.2 ± 0.5	0.264
29	3	27.3 ± 0.5	0.112
30	4	24.8 ± 0.6	0.003
31	6	27.7 ± 4.0	0.612
32	4	25.1 ± 0.9	0.021

Table 5.7: Identification of Col 4x submissive BC2(Tsu-1)F_2 plants. BC2(Tsu-1)F_2 plants were crossed with Col 4x pollen and the mean seed weight of the resulting seeds determined. P values are for a comparison between [BC2(Tsu-1)F_2 X Col 4x] and the control cross, [Tsu 2x X Col 4x]**.** The plant selected for entry into the next round of back-crossing is highlighted in yellow

Cross	Number of Siliques	Mean seed weight (µg) ± se	P value
[Col 2x X Col 4x]	4	17.1± 1.2	
BC2(Tsu-1)F2 X Col 4x			
1	4	23.4 ± 2.1	0.033
2	4	26.2 ± 1.4	0.003
3	4	31.2 ± 2.3	0.002
4	4	29.5 ± 0.7	0.001
5	3	19.9 ± 3.5	0.434
6	4	29.5 ± 1.4	0.001
7	4	15.9 ± 2.8	0.641
8	3	24.9 ± 2.0	0.019
9	4	15.7 ± 2.8	0.658
10	3	27.1 ± 0.9	0.002
11	4	30.9 ± 1.3	0.001
12	4	26.1 ± 1.2	0.002
13	4	25.3 ± 0.9	0.002
14	4	12.1 ± 1.8	0.004
15	3	26.0 ± 0.3	0.002
16	4	20.5 ± 0.7	0.053
17	3	28.2 ± 0.3	0.001
18	4	26.1 ± 0.4	0.001
19	4	27.6 ± 1.0	0.001
20	3	31.1 ± 0.7	0.001
21	4	23.4 ± 2.0	0.039
22	3	29.3 ± 0.7	0.001
23	4	32.4 ± 0.7	0.001
24	4	8.1 ± 1.6	0.003
25	4	29.4 ± 0.4	0.001
26	4	27.2 ± 1.7	0.003
27	4	27.6 ± 1.4	0.001
28	4	33.1 ± 1.1	0.001
29	3	31.7 ± 0.0	0.001
30	4	27.7 ± 1.7	0.002
31	4	13.3 ± 2.2	0.165
32	4	20.3 ± 3.8	0.464
33	3	16.4 ± 0.9	0.668
34	3	15.6 ± 1.2	0.424

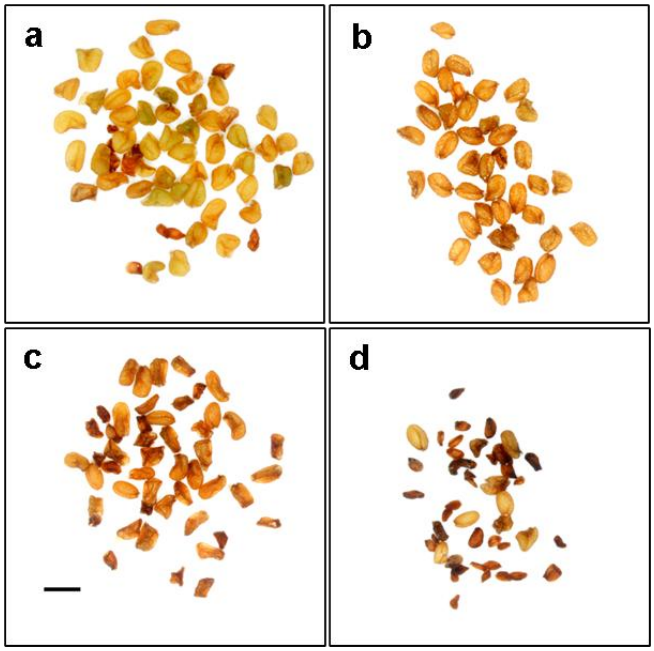

Figure 5.20: Seed produced in [BC2F$_2$XCol4x] test crosses. (a) [Tsu-1 X Col 4x] control seed (b) seed from a highly resistant plant (c) [Col2x X Col 4x] control seed (d) seed from a highly submissive plant. Scale bar = 1mm.

5.2.3.4. Identification of resistant BC2(Col-0)F$_3$ and submissive BC2(Tsu-1)F$_3$ plants

The resistant and submissive BC2(Col-0)F$_2$ plants selected as described in the previous were selfed pollinated to produce BC2(Col-0)F$_3$ seed.

A total of 23 BC2(Col-0)F$_3$ plants were produced from seed from the highly resistant plant BC2(Col-0)F$_2$ (number 26: Table 5.6), and the mean seed weight determined for each following a cross to 4x Col-0 (Table 5.8). Almost half of plants (11 from 23) had mean seed weights that were not significantly different to the [Tsu 2x X Col 4x] control ($P > 0.05$), indicating high frequency inheritance and/or dominance of the modifier trait from the Tsu-1 parent. An example of seed from a highly resistant BC2(Col-0)F$_3$ plant (number 6; Table 5.8) is shown in Figure 5.21b. This plant had an exceptionally high mean seed weight and good seed morphology, and was the selected for entry into the next round of backcrossing.

A total of 16 BC2(Tsu-1)F$_3$ plants were grown from selfed seed of the selected highly submissive BC2(Tsu-1)F$_2$ plant (number 24: Table 5.7) and crossed with Col-0 4x pollen to determine the mean weight of seeds (Table 5.9). Approaching 50% of plants (8 from 16) had mean seed weights that were not significantly different to the [Col 2x X Col

4x] control (P > 0.05). An example of seed from a highly submissive BC2(Tsu-1)F_3 plant (number 6; Table 5.9) is shown in Figure 5.21d. This plant met the selection criteria and was entered into the next round of backcrossing.

Table 5.8 Identification of Col 4x resistant BC2(Col-0)F_3 plants. BC2(Col-0)F_3 plants were crossed with Col 4x pollen and the mean seed weight of the resulting seeds determined. P values are for a comparison between [BC2(Col-0)F_3X Col 4x] and the control cross, [Tsu 2x X Col 4x]**.** The plant selected for entry into the next round of back-crossing is highlighted in blue.

Cross	Number of Siliques	Mean seed weight (µg) ± se	P value
[Tsu 2x X Col 4x]		33.3 ± 1.1	
BC2(Col-0)F3 X Col4x			
1	4	29.7 ± 1.0	0.042
2	5	24.3 ± 1.0	0.001
3	5	35.2 ± 1.8	0.376
4	6	39.4 ± 2.9	0.004
5	3	34.0 ± 3.5	0.868
6	5	54.6 ± 1.5	0.001
7	5	34.7 ± 0.1	0.284
8	6	39.1 ± 2.4	0.066
9	6	33.2 ± 2.6	0.978
10	4	27.7 ± 5.6	0.393
11	5	41.3 ± 1.6	0.005
12	5	40.9 ± 1.4	0.004
13	5	45.5 ± 1.2	0.001
14	4	39.3 ± 2.7	0.051
15	4	38.8 ± 1.6	0.119
16	5	48.4 ± 1.6	0.001
17	5	50.4 ± 0.8	0.001
18	5	44.4 ± 2.2	0.008
19	5	43.6 ± 2.8	0.022
20	3	39.6± 1.3	0.047
21	5	35.2 ± 1.8	0.379
22	4	30.6 ± 2.5	0.375
23	5	39.0 ± 2.1	0.058

Table 5.9: Identification of Col 4x submissive BC2(Tsu-1)F_3 plants. BC2(Tsu-1)F_3 plants were crossed with Col 4x pollen and the mean seed weight of the resulting seeds determined. P values are for a comparison between [BC2(Tsu-1)F_3 X Col 4x] and the control cross, [Tsu 2x X Col 4x]**.** The plant selected for entry into the next round of back-crossing is highlighted

Cross	Number of Siliques	Mean seed weight (µg) ± se	P value
[Col 2x X Col 4x]	3	23.5 ± 3.0	
BC2(Tsu-1)F3 X Col 4x			
1	4	30.2 ± 1.0	0.059
2	4	39.7 ± 1.7	0.004
3	4	38.0 ± 3.3	0.031
4	3	35.9 ± 1.3	0.019
5	4	28.0 ± 0.9	0.161
6	5	22.1 ± 1.5	0.692
7	3	52.4 ± 3.2	0.003
8	3	31.0 ± 4.0	0.205
9	4	35.5 ± 2.2	0.021
10	3	39.8 ± 4.7	0.043
11	4	41.3 ± 3.3	0.012
12	5	22.2 ± 1.2	0.676
13	4	39.9 ± 2.9	0.012
14	4	31.2 ± 3.3	0.159
15	5	27.6 ± 0.4	0.723
16	3	25.2 ± 2.1	0.666

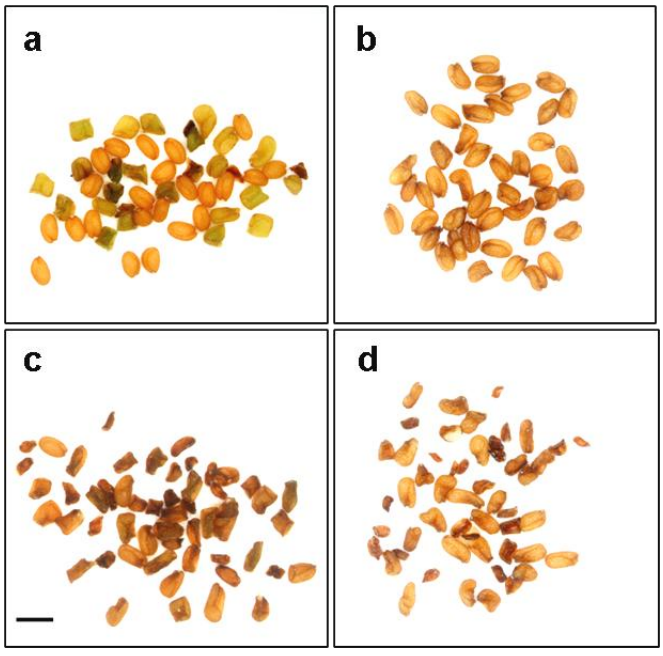

Figure 5.21: Seed produced in [BC2F_3XCol4x] test crosses. (a) [Tsu X Col 4x] control seed (b) seed from a highly resistant plant (c) [Col 2x X Col 4x] control seed (d) seed from a highly submissive plant. Scale bar = 1mm.

5.2.3.5. Identification of resistant BC3(Col-0)F_3 and submissive BC3(Tsu-1)F_3 plants

The final step in producing the resistant BC3(Col-0)F_3 and BC3(Tsu-1)F_3 was to perform a further backcross and selection.

A total of 31 BC2(Col-0)F_3 plants were produced from seed from the highly resistant plant BC2(Col-0)F_3 (number 6: Table 5.8), and the mean seed weight determined for each following a cross to 4x Col-0 (Table 5.10). The majority of plants (28 from 31) had mean seed weights that were not significantly different to the [Tsu 2x X Col 4x] control ($P > 0.05$), indicating high frequency inheritance and/or dominance of the modifier trait from the Tsu-1 parent. An example of seed from a highly resistant BC3(Col-0)F_3 plant (number 18; Table 5.10) is shown in Figure 5.22b. This plant had an exceptionally high mean seed rate and good seed morphology, and was the selected for mapping.

A total of 25 BC3(Tsu-1)F_3 plants were grown from selfed seed of the selected highly submissive BC2(Tsu-1)F_2 plant (number 6: Table 5.9) and crossed with Col 4x pollen to determine the mean weight of seeds (Table 5.11). More than 50% of plants (13 from 25) had mean seed weights that were not significantly different to the [Col 2x X Col 4x] control ($P > 0.05$). An example of seed from a highly submissive BC3(Tsu-1)F_3 plant (number 3; Table 5.11) is shown in Figure 5.22d. This plant had an exceptionally low mean seed weight and was therefore considered suitable for mapping.

Table 5.10: Identification of Col 4x resistant BC3(Col-0)F_3 plants. BC3(Col-0)F_3 plants were crossed with Col 4x pollen and the mean seed weight of the resulting seeds determined. P values are for a comparison between [BC3(Col-0)F_3X Col 4x] and the control cross, [Tsu 2x X Col 4x]**.** The plant selected for entry into the next round of back-crossing is highlighted

Cross	Number of Siliques	Mean seed weight (µg) ± se	P value
[Tsu 2x X Col 4x]	3	31.3 ± 1.2	
BC3(Col-0)F3 X Col4x			
1	6	33.5 ± 1.8	0.366
2	6	31.7 ± 1.3	0.851
3	6	28.0 ± 1.1	0.101
4	6	28.9 ± 1.4	0.323
5	5	32.4 ± 1.8	0.674
6	6	30.7 ± 0.4	0.572
7	3	32.7 ± 1.3	0.464
8	6	34.1 ± 0.3	0.016
9	6	30.2 ± 1.1	0.565
10	4	26.9 ± 1.3	0.069
11	6	28.4 ± 0.6	0.041
12	6	28.8 ± 1.0	0.173
13	3	29.8 ± 1.0	0.391
14	3	29.4 ± 1.2	0.343
15	5	31.2 ± 0.9	0.993
16	5	29.4 ± 1.2	0.353
17	4	33.2 ± 0.4	0.145
18	5	38.7 ± 2.5	0.077
19	5	33.7 ± 0.5	0.081
20	4	34.3 ± 0.2	0.229
21	4	29.9 ± 1.1	0.467
22	3	31.4 ± 1.5	0.958
23	4	31.8 ± 0.4	0.659
24	4	32.4 ± 0.3	0.343
25	3	31.5 ± 1.1	0.871
26	4	31.2 ± 1.4	0.965
27	4	26.4 ± 0.7	0.014
28	4	29.1 ± 0.3	0.106
29	4	30.5 ± 0.9	0.631
30	4	29.6 ± 0.5	0.215
31	3	33.0 ± 0.9	0.331

Table 5.11: Identification of Col 4x submissive BC3(Tsu-1)F_3 plants. BC3(Tsu-1)F_3 plants were crossed with Col 4x pollen and the mean seed weight of the resulting seeds determined. P values are for a comparison between [BC3(Tsu-1)F_3 X Col 4x] and the control cross, [Col 2x X Col 4x]**.** The plant selected for entry into the next round of back-crossing is

Cross	Number of Siliques	Mean seed weight (µg) ± se	P value
[Col 2x X Col 4x]	3	21.3 ± 0.5	
BC3(Tsu-1)F3 X Col 4x			
1	3	10.4 ± 2.3	0.011
2	4	33.4 ± 2.3	0.007
3	3	8.5 ± 2.2	0.005
4	3	9.3 ± 1.6	0.002
5	3	23.2 ± 1.8	0.369
6	5	24.2 ± 1.4	0.165
7	3	32.7 ± 0.8	0.001
8	4	12.5 ± 1.9	0.012
9	3	12.3 ± 2.4	0.021
10	5	18.8 ± 3.1	0.568
11	3	30.4 ± 0.3	0.001
12	4	23.9 ± 3.4	0.486
13	3	13.5 ± 4.0	0.128
14	5	24.2 ± 4.9	0.582
15	3	16.5 ± 3.0	0.244
16	3	32.7 ± 0.5	0.001
17	3	25.0 ± 0.5	0.609
18	3	18.4 ± 3.4	0.509
19	4	24.5 ± 3.9	0.453
20	6	14.2 ± 4.4	0.189
21	5	22.6 ± 4.7	0.795
22	4	34.3 ± 3.2	0.023
23	3	25.2 ± 4.9	0.532
24	3	9.5 ± 1.0	0.001
25	3	33.5 ± 1.3	0.001

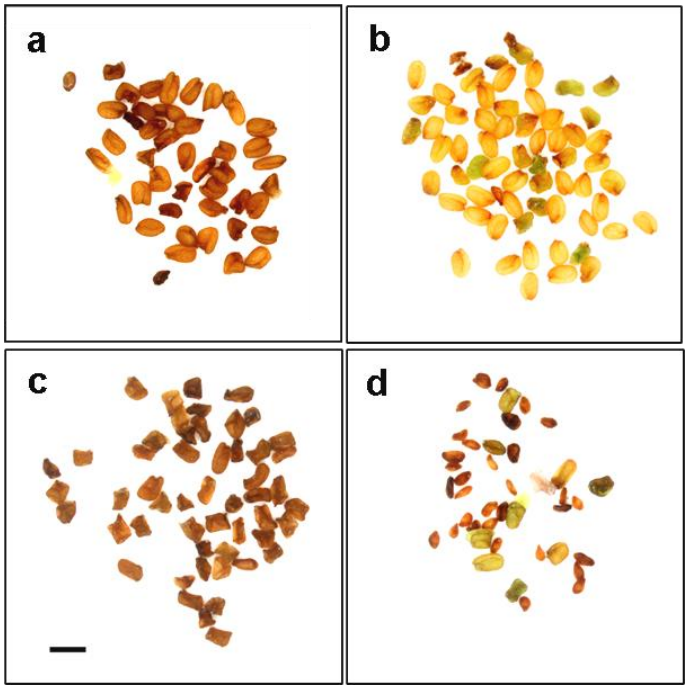

Figure 5.22: Seed produced in [BC3F$_3$XCol4x] test crosses. (a) [Tsu-1 X Col 4x] control seed (b) seed from a highly resistant plant (c) [Col 2x X Col 4x] control seed (d) seed from a highly submissive plant. Scale bar = 1mm.

5.2.4. Molecular genetic evaluation of the BC3F$_3$ populations

As stated earlier (5.1.2) the objective of the recurrent backcrossing programme was to generate material suitable for mapping the Tsu-1 maternal modifier by the backcross-based method. To evaluate progress toward this goal, the BC3F$_3$ material was subjected to molecular genetic analysis using CAPS markers to determine the relative content of parental DNA. The expectation was that BC3(Col-0)F$_3$ plants would have a high content of Col-0 DNA, and therefore Col-0 markers, whilst Tsu-1 markers would dominate in the DNA of BC3(Tsu-1)F$_3$ plants. An initial evaluation used 4 markers per chromosome, split between the two arms (Figure 5.23; Tables 2.3 and 2.4).

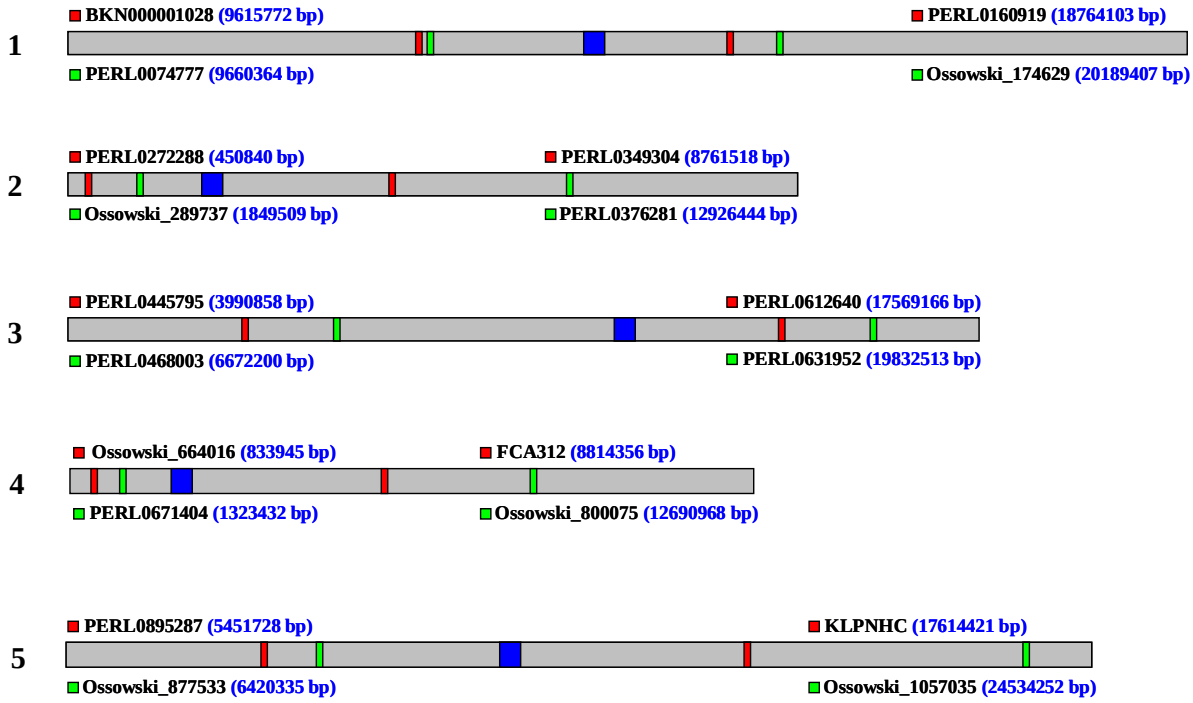

Figure 5.23: Physical chromosome map of *A. thaliana* showing the positions of CAPS markers used to evaluate the BC3F$_3$ population
The name and start position in bp of the chromosome are indicated.
Blue box shows the position of centromere.

5.2.4.1. Marker analysis of BC3(Col-0) F$_3$ progenies

A total of three highly resistant BC3(Col-0)F$_3$ plants were subjected to CAPS marker analysis. Figure 5.24 shows the results for the chromosome 1 markers to illustrate the ease of scoring. For this chromosome, all three plants were scored Col-0 for each marker. The complete set of results is shown in Table 5.12. The data reveals variation for the frequency of Col-0 verses Tsu-1 markers across the chromosomes. Chromosomes 1 and 4 had the highest frequency of markers derived from the recurrent parent, which was Col-0 in this instance: 100% (24 from 24) and 96% (23 from 24), respectively. In contrast, the frequency of Col-0 markers for chromosome 5 was relatively low: 62.5% (15 from 24). The total frequency of Col-0 markers was 82% (98 from 120) indicating that as expected BC3(Col-0) F$_3$ plants were enriched for Col-0 DNA.

Table 5.12: Evaluation of BC3(Col-0)F_3 plants using molecular markers.
Markers 1-4 are as shown in Figure 5.23, and are numbered in the Table consecutively from north to south. Three plants were scored for each marker. The number of plants that were Col-0, Tsu-1 or heterozygous was recorded for each marker

Markers	Chromosome 1			Chromosome 2			Chromosome 3			Chromosome 4			Chromosome 5		
	Col	Tsu	Het	Col	Tsu	Het	Col	Tsu	Het	Col	Tsu	Het	Col	Tsu	Het
1	3	0	0	0	0	3	3	0	0	3	0	0	0	0	3
2	3	0	0	3	0	0	3	0	0	3	0	0	0	0	3
3	3	0	0	3	0	0	3	0	0	3	0	0	0	0	3
4	3	0	0	0	0	3	0	3	0	2	0	1	3	0	0

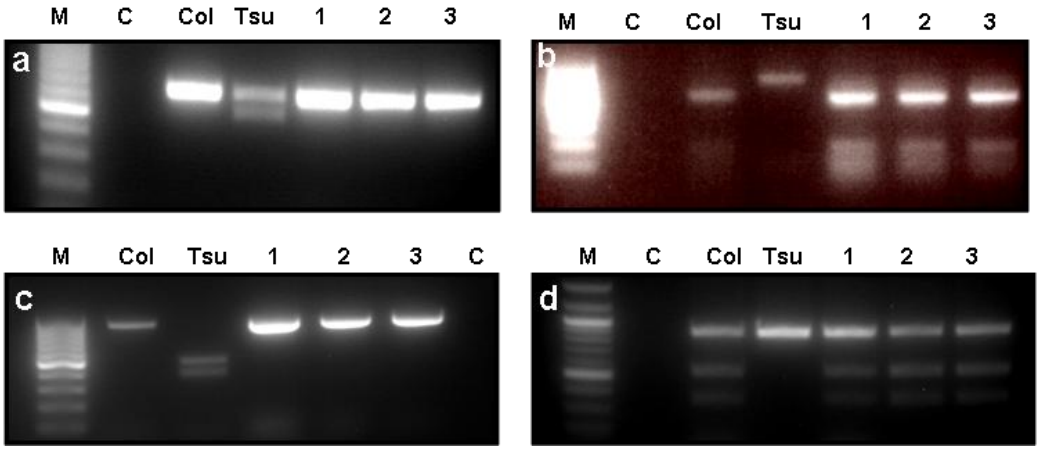

Figure 5.24: Analysis of BC3(Col-0)F_3 DNA using chromosome 1 molecular markers. DNA from three plants was subjected to CAPS marker analysis and the products visualised by gel electrophoresis. The chromosome 1 markers were: a) BKN000001028; b) PERL0160919; c) PERL0074777; d) Ossowski_174629 as shown in Figure 5.23. The lanes contain: M = DNA ladder (100 bp); C = Control (H_2O); Col = Col-0 DNA; Tsu = Tsu-1 DNA; 1, 2, 3 = DNA from BC3(Col-0)F_3 plants.

5.2.4.2. Marker analysis of BC3(Tsu-1) F_3 progenies

Highly submissive BC3(Tsu-1)F_3 plants were subjected to the same CAPS marker analysis as described in the previous section. Figure 5.25 shows the results for the chromosome 4 markers. For this chromosome, three plants were scored Col-0 for all markers. The complete set of results is shown in Table 5.13. As with BC3(Col-0)F_3 above, the data reveals some variation in the frequency of Col-0 verses Tsu-1 markers across the chromosomes. Again chromosomes 1 and 4 had the highest frequency of markers derived from the recurrent parent, Tsu-1 in this instance: 100% (24 from 24) in both cases. However, in contrast to BC3(Col-0)F_3, chromosomes 2, 3 and 5 also had a high frequency of Tsu-1 markers: 23% (23 from 24); 83% (20 from 24); and 86% (21 from 24), respectively. The total frequency of Tsu-1 markers was 93% (112 from 120) indicating that the BC3(Tsu-1) F_3 plants were highly enriched for Tsu-1 DNA.

Table 5.13: Evaluation of BC3(Tsu-1)F_3 plants using molecular markers
Markers 1-4 are as shown in Figure 5.23, and are numbered consecutively from north to south. Three plants were scored for each marker. The number of plants that were Col-0, Tsu-1 or heterozygous was recorded for each marker.

	Chromosome 1			Chromosome 2			Chromosome 3			Chromosome 4			Chromosome 5		
Markers	**Col**	**Tsu**	**Het**	**Col**	**Tsu**	**Het**	**Col**	**Tsu**	**Het**	**Col**	**Tsu**	**Het**	**Col**	**Tsu**	**Het**
1	0	3	0	0	3	0	0	3	0	0	3	0	0	3	0
2	0	3	0	0	3	0	0	3	0	0	3	0	0	3	0
3	0	3	0	0	3	0	2	1	0	0	3	0	0	3	0
4	0	3	0	0	2	1	0	3	0	0	3	0	0	0	3

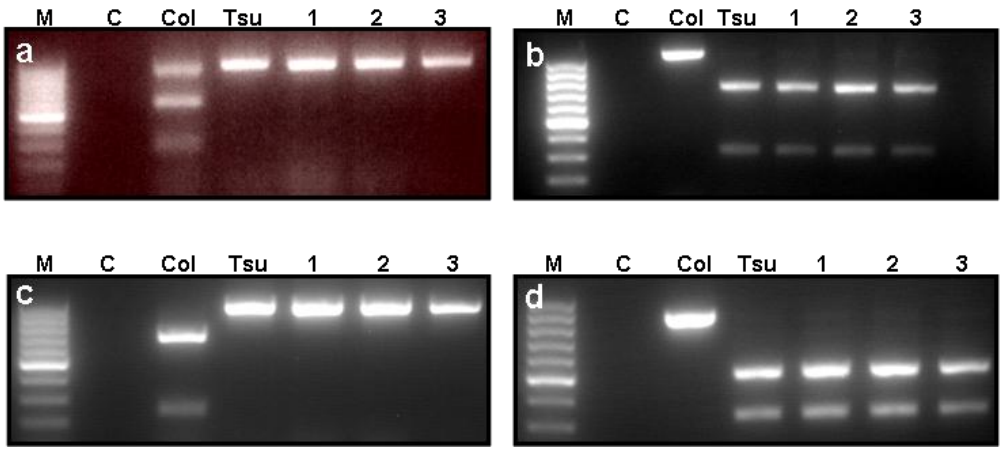

Figure 5.25: Analysis of BC3(Tsu-1)F_3 DNA using chromosome 4 molecular markers. DNA from three plants was subjected to CAPS marker analysis and the products visualised by gel electrophoresis. The chromosome 4 markers were: (a)Ossowski_664016 marker (b) PERL0671404 marker (c) FCA312 marker (d) Ossowski_800075 marker as shown in Figure 5.23. The lanes contain: M = DNA ladder (100 bp); C = Control (H_2O); Col = Col-0 DNA; Tsu = Tsu-1 DNA; 1, 2, 3 = DNA from BC3(Tsu-1)F_3 plants.

5.3. Discussion

Numerous plant researchers (Ramsey and Schemske, 1998; Otto and Whitton, 2000; Soltis *et al*., 2007, and Dilkes *et al*., 2008) have proposed that postzygotic lethality barriers are one of the most important mechanisms by which shifts in ploidy can promote the process of speciation. Significantly, considerable variability in this trait exists within species and between species (Leblanc *et* al., 2002 and Dilkes *et al*., 2008). *A. thaliana* exhibits genetic variation for postzygotic lethality (Chapter 4). Specifically, whilst most ecotypes produced plump seed after an intra-ecotype [2x X 4x] cross, two ecotypes, Col-0 and RLD, this cross resulted in a high frequency of shriveled seed. These ecotypes were thus identified as carrying an unusual killing activity that caused triploid block, which in the case of the Columbia ecotype, was termed Col-killing. Crosses between a panel of diploid *A. thaliana* ecotypes and a Col 4x pollen parent, revealed genetic variation also existed for Col-killing between ecotypes. Some ecotypes behaved as Col-0, suffering similarly high levels of seed lethality, whereas others, such as C24 and Tsu-1 almost completely resisted Col-0-killing activity to produce near 100% viable seed. These ecotypes appeared to possess maternal 'modifiers' that somehow resist the Col-0 killer. The present chapter was an attempt at the phenotypic and genetic characterization of the Tsu-1 maternal modifier.

5.3.1. The Tsu-1 maternal modifier resists Col-killing by promoting endosperm cellularisation in [2x X 4x] seed

The endosperm is a fertilisation product and an important component of seed. The earliest evidence of aberrant development in the progeny of paternal excess crosses was visible in the endosperm (Scott *et al*., 1998a; Dilkes *et al*., 2008).

In crosses between plants of different ploidy levels, endosperm mutilation commonly occurs, often preventing successful hybridisation. In our study following the interploidy crosses, we found that the paternal excess crosses of both diploid ecotypes Tsu-1 and Col-0 with the tetraploid Col-0 differed in their respective endosperm growth phenotypes, as both crosses on their basis of genome frequency showed 3x embryos (1m:2p) and 4x endosperms (2m:2p). However, doubling the paternal genome of Col-0 in its interploidy crossing with both diploids of Tsu-1 and Col-0 showed increase in the embryo sac area and both chalazal endosperm and nodules size more than that observed in [Tsu 2x X Col 2x] (Figures 5.1 and 5.2). Our findings suggest that, both interploidy crosses

of [Tsu 2x X Col 4x] and [Col 2x X Col 4x] exhibited delayed cellularisation of the endosperm and probably they showed acceleration in mitosis process which results in developing large seeds at maturity as declared by Scott *et al*., (1998a). However, at 6 and 7 DAP the large differences in embryo sac area measurement was obvious in [Col 2x X Col 4x] than both crosses [Tsu 2x X Col 2x] and [Tsu 2x X Col 4x]. These observations indicate that the Tsu-1 seed parent restricts over-proliferation of the endosperm normally induced by a Col 4x pollen parent. The data supports the observations that both final size and viability of a seed reveal the effectiveness of the endosperm in provisioning the developed embryo (Scott *et al*., 1998a). Nevertheless, both chalazal endosperm and nodules areas from [Col 2x X Col 4x] cross was no smaller than that of [Tsu 2x X Col 4x] mainly at 6 and 7 DAP as demonstrated in (Figures 5.1 and 5.3); however, their size were considerably larger than the normal diploid cross between Tsu-1 and Col-0. This data showed that considerable chalazal endosperm over-growth occurs in the rescuing [Tsu 2x X Col 4x] cross, but was not related to seed failure as occurs in the [Col 2x X Col 4x] cross. This indicates that rescue of lethality by Tsu-1 was not associated with the resolution of all abnormal features of the endosperm. For example, neither the size of the chalazal endosperm nor the presence of nodules in the peripheral endosperm was affected by Tsu-1 providing rescue of lethality. Similar observations were observed with *DSL1*^{Ler} and *ttg2* mutants in rescuing of lethality with paternal Col 4x (Dilkes *et al*., 2008). Also, Haughn and Chaudhury (2005) showed that the reduced growth of maternal tissues in *ttg2* mutants restricts embryo sac expansion and promotes endosperm cellularisation.

Cellularisation also appears to be the critical feature in paternal excess interploidy crosses that determines the development of the embryo. Thus, cellularised peripheral endosperm was observed more frequently at 6 and 7 DAP in [Tsu 2x X Col 2x] and at the middle of 7 DAP in [Tsu 2x X Col 4x] than in [Col 2x X Col 4x] which showed no cellularisation. This suggests that reduced growth of maternal tissues in Tsu-1 restricts embryo sac expansion and promotes cellularisation of the endosperm; while, Col-0 showed over-growth and ceases endosperm cellularisation. However, same suggestion was demonstrated by Dilkes *et al*., (2008) in their research studies for both *DSL1* and *ttg2* rescuing mutants in that the peripheral endosperm cellularisation was always greater in backgrounds that increased seed survival.

5.3.2. The Tsu-1 maternal modifier behaves as a genetically dominant trait

In our research studies involving both ecotypes Tsu-1 and Col-0, we found that both ecotypes behave differently when they are crossed with paternal Col 4x. The frequency of shriveled seeds was higher in [Col 2x X Col 4x] cross than that observed in [Tsu 2x X Col 4x] cross as shown in Figure 5.6. However, the use of mean seed weight was intended to overcome any potential problems with the subjective nature of the shrivelled seed measure. Our findings showed that the mean seed weight in [Tsu-1 X Col 4x] was higher than [Col 2x X Col 4x] which indicates higher frequency of plump seeds resulting from [Tsu-1 X Col 4x] cross (Figure 5.7). This phenomenon suggests that Tsu-1 acts as a strong maternal modifier of Col-induced seed killing. However, it confirms the occurrence of a specific factor (gene or genes) within Tsu-1 ecotype that has the ability to resist the killing activity caused by paternal excess of Col 4x. Similarly, Dilkes *et al*., (2002) have stated that within particular genotypes, crosses between diploid and tetraploid lines can result in lethality, and have identified this lethality cause to involve a pathway adapted by the maternal effect transcription factor *TRANSPARENT TESTA GLABRA2* (*TTG2*).

The genetic basis of the Tsu-1 modifier using a recurrent backcrossing method to obtain a $BC3F_3$ generation as shown in Figure 5.9 revealed the dominancy of the modifier trait. However, the ability of the F_1 plants generated from a cross between diploid Tsu-1/Col-0 to resist Col-killing using Col 4 x pollen confirmed that Tsu-1 modifier is a dominant trait (Figure 5.11). Similarly, Dilkes *et al*., (2008) showed that the maternal contribution in L*er* alleles in *DSL* (*Dr. Strangelove 1*) was sufficient to rescue part of lethality in [F_1 X Col 4x] crosses which explains that gene regulation during life cycle stages is correlated with variation in seed fitness, parent-of-origin effects and hybridisation barriers, and not only the genomic imprinting in the zygotes. In the present study, a progeny test of F_2 plants was also conducted to test their homozygosity to study the genetics of the Tsu-1 modifier. This is to perform the recurrent backcrossing strategy by selecting the most 4x Col-0 resistant and submissive F_2 plants. There was a variation in the frequency of plump/shrivelled seed across the population of [F_2 X Col 4x] plants (Figure 5.14). However, this indicates that F_2 plants were either highly resistant to Col 4x, similar

to Tsu-1; (Figure 5.15), or highly submissive to 4x Col-0 pollen, similar to Col-0; (Figure 5.16).

5.3.3. Recurrent backcrossing produces plants with potential for mapping the Tsu-1 maternal modifier

The molecular back-cross (BC) breeding strategy being adapted should be able to help in understanding the process of Tsu-1 maternal modifier mapping. Thus, the advanced back-cross approach appeared to be an appropriate method to accelerate the process of introgressing interested traits or genes into a selected material (Robin *et al*., 2003).

To map the Tsu-1 modifier gene, a number of recurrent backcrosses (BCs) were applied using the highly resistant and submissive F_2 plants. Thus, the backcrossing (BC) method is widely recognized as a powerful tool to study different inherited traits as well as QTLs (Butruille *et al*., 1999). In this study, we generated a number of $BC3F_3$ plants starting from highly resistant and highly submissive F_2 population. Further analysis for both highly resistant and submissive progenies was done through three successive BCs (BC1, BC2, and BC3); however, the aim was to produce a resistant plant BC3(Col-0)F_3 that would consist of a little amount of Tsu-1 DNA carrying the Tsu-1 modifier locus, and a relatively large amount Col-0 DNA. On the other hand, the submissive plant BC3(Tsu-1)F_3 having a small amount of Col-0 DNA carrying the Col-0 modifier locus, and a relatively large amount Tsu-1 DNA.

The molecular genetic analysis of $BC3F_3$ plants using CAPS markers to determine the relative content of parental DNA showed that the BC3(Col-0) F_3 plants were Col-0 DNA as they displayed high frequency of Col-0 markers (82 %) (Table 5.12), while BC3(Tsu-1) F_3 plants were Tsu-1DNA background showing (93 %) high frequency of Tsu-1 markers (Table 5.13). This method describes the efficiency of transferring a genetic or multigenic character through back-cross breeding methods.

Similarly, several molecular mapping studies by many biologists have been applied and adapted to different useful crop plants using back-crossed populations in order to identify and map different traits. For instance, Robin *et al*., (2003) mapped the osmotic adjustment trait associated with drought tolerance in rice from a number of different ($BC3F_3$) families using an advanced back-cross inbred lines via an indica donor, IR62266-42-6-2, to introgress osmotic adjustment into a selected japonica cultivar, IR60080-46A. Moreover, larger magnitudes of segregation distortion were observed in other back-crossed

inbred lines (BIL) populations for the reason that they involved interspecific crosses, and because selection was practiced in favor of the recurrent parent (Fulton *et al*., 1997; Lin *et al*., 1998; and Robin *et al*., 2003). Until recently the effectiveness of phenotypic selection for different traits in the BC progenies remains the key to success for many molecular mapping approaches.

5.4. Conclusion

The Tsu-1 ecotype is highly resistant to 4x Col-0 killing. A detailed examination of seed development showed that Tsu-1 rescues Col-induced lethality despite not preventing substantial endosperm over-growth. The basis of rescue was successful cellularisation of the endosperm, as previously described for L*er*- and *ttg2*-mediated rescue (Dilkes *et al.*, 2008). In preparation for mapping the Tsu-1 modifier locus a reliable phenotype scoring was established based on mean seed weight. This measure was positively correlated with a high frequency of plump seed and therefore presence of the Tsu-1 allele of the proposed rescuing locus. Genetic analysis established that modifier trait was apparently dominant. The mapping strategy adopted was to perform a series of recurrent backcrosses starting from a pair of highly resistant and highly submissive F_2 plants. Backcrossing produced both a resistant BC3(Col-0)F_3 and a submissive BC3(Tsu-1)F_3 plants. Preliminary DNA marker analysis established that the resistant BC3(Col-0)F_3 was enriched for Col-0 DNA and sensitive BC3(Tsu-1)F_3 was enriched for Tsu-1 DNA. These populations will be useful in identifying Tsu-1 maternal modifier genes.

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Chapter 6

6. The genetics of the Col-0 killing trait

6.1. Introduction

Most studies of plant hybridisation have been concerned with documenting its occurrence in different plant groups. Thus, numerous studies have been performed on crossing between plants, and substantial evidence has been gathered on the kinds and strength of post-mating reproductive barriers in both closely and distantly related plant species (Grant, 1981; Jackson, 1985; Rieseberg and Carney, 1998).

6.1.1. Hybridisation barriers control F_1 lethality in the Col-0 ecotype

One of the most interesting aspects of speciation research is to understand the molecular mechanisms underlying the lethality of F_1 hybrids that occurs following crosses between diverged parents. Whilst most post-mating barriers occur between species (Rieseberg and Carney, 1998), crosses between diploid and tetraploid individuals of the same species frequently result in the lethality of F_1 hybrids; a process termed triploid block (Köhler *et al*., 2010). Such postzygotic lethality is hypothesized to play an important role in polyploidy speciation (Dilkes *et al.*, 2008).

Chapter 4 describes an investigation of F_1 lethality in interploidy crosses within *A. thaliana*. In contrast to many other species, most ecotypes of *A. thaliana* that were tested did not exhibit triploid block i.e. diploid-tetraploid interploidy crosses produced very low levels of aborted seed. However, in contrast, the Columbia ecotype exhibited an unusual paternal-only triploid block where the maternal excess cross, [Col 4x X Col 2x] produced mostly viable seed whilst the reciprocal, paternal excess cross [Col 2x X Col 4x] resulted in high levels of seed abortion (Dilkes *et al.*, 2008). Columbia is therefore more representative of other species in its interploidy crossing behaviour than the bulk *A. thaliana* ecotypes.

A number of authors have hypothesized that F_1 lethality barriers resulting from both interploidy and interspecific crosses and changes in genome dosage could be controlled by the same mechanisms (Johnston *et al.*, 1980; Haig and Westoby, 1991; Bushell *et al.*, 2003, and Dilkes *et al.*, 2008). One of these mechanisms is the variation in imprinted genes, which is responsible for the postzygotic lethality in interploidy crosses (Lin 1984; Haig and Westoby, 1991, and Dilkes *et al.*, 2008). The interploidy and interspecific

barriers are proposed to be affected by genome dosage, multiple dosage-sensitive genetic mechanisms, and inclusive of imprinting (Johnston *et al.*, 1980; Haig and Westoby, 1989; Haig and Westoby, 1991; von Wangenheim and Peterson, 2004; Dilkes and Comai, 2004, and Dilkes *et al.*, 2008). The identification of Columbia as having a typical interploidy hybridisation barrier i.e. triploid block, at least on the paternal side, provided an opportunity to investigate postzygotic lethality at the molecular genetic level, and thereby provide evidence for or against the above mechanisms.

6.1.2. STAIRS and CSS lines: powerful genetic resources for functional genomic studies in *Arabidopsis*

Many researchers found that different biological and ecological quantitative traits in plants and animals are controlled by a serious of genes that shows continuous variation in the progeny. These genes are known as quantitative trait loci (QTL). Many efforts have been made to map such QTL because of their biological and agronomic importance (Takahashi *et al.*, 2001). The development of high-density linkage maps based on DNA markers has facilitated the mapping of QTL to high resolution (Paterson *et al.*, 1988; Takahashi *et al.*, 2001). However, the main problem in studying QTL remains providing sufficient resolution to subsequently identify underlying candidate genes such as that in rice and Arabidopsis (Zeng *et al*., 2007) and potato (Kloosterman, 2010).This problem is mainly due to the relatively small effect of the allelic differences (their individual effects on the phenotype are very small) compared to the large variation caused by other loci and the environment.

Chromosome Substitution Strains (CSS) and Stepped Aligned Inbred Recombinant Strains (STAIRS) are two associated genetic resources in *A. thaliana* that are useful in mapping QTL (Koumproglou *et al.*, 2002). The CSS lines are a series of five homozygous strains (CSS1-5), in which one of the Col chromosome pairs has been replaced by the corresponding L*er* chromosome. For example, in CSS1 chromosome 1 is derived from L*er* and chromosomes 2-5 from Col. These strains allow genetic differences between Col and L*er* to be assigned easily to particular chromosomes which facilitates the QTL to be linked in repulsion and not cancel out (Koumproglou *et al.*, 2002). CSS lines have been used in diverse species such as wheat (Law *et al.*, 1983; 1987), rice (Wang *et al.*, 2006; Zhu *et al*., 2009) and the mouse (Nadeau *et al.*, 2000; Singer *et al.*, 2005; Burrage *et al.*, 2010).

STAIRS are derived from each CSS line and are used to progress mapping toward gene location. They consist of a large number of lines; each line consists of a homozygous chromosome (known as single recombinant lines or SRLs) with a single crossover, such that the chromosome will contain the donor genes at one end and the recipient genes at the other end (Koumproglou *et al.*, 2002). In fact, STAIRS allow genetic differences between donor and recipient individuals to be allocated to particular regions within a chromosome. SRLs are stacked chronologically for each chromosome in a step-like progression with each successive line from both donor and recipient individuals. STAIRS with wide steps allow rough gene mapping (5-10 cM), while narrower steps within that region allow gene location to less than 1 cM. The major advantage of STAIRS is that they enable comparisons of the phenotypes of numbers or pairs of lines in which the genetic differences lie in short regions of one selected chromosome. Moreover, CSS lines and STAIRS have a particular value in the analysis of QTLs, for candidate gene searching, and for different gene expression studies.

6.1.3. Recombinant inbred lines (RILs) and their role in *Arabidopsis* genomic studies

Many agriculturally important traits such as yield, quality and some forms of disease resistances are controlled by QTL (Collard *et al*., 2005). Most QTL detection experiments have been conducted starting with inbred lines including, backcross (BC), advanced backcross, F_2, recombinant inbred (RI) populations, intermated recombinant inbred (IRI) populations, advanced intercross (AI) populations, advanced backcross populations, double haploid (DH) populations (Winkler *et al.*, 2003; Broman, 2005; Kao, 2006). *A. thaliana* recombinant inbred lines (RILs) can serve as powerful tools for genetic mapping studies. The primary advantage of RI populations is that is that they can be used indefinitely in mapping since each RIL is an inbred strain and can be easily propagated; hence, these populations can be evaluated by different researchers, in different environments, and at different times (Wu *et al.*, 2003). Many researchers have used RILs populations for mapping research a variety of plants including maize (Burr *et al.*, 1988; Burr and Burr, 1991; Carson *et al*., 1996; Austin and Lee, 1996), wheat (Snape *et al.*, 1985; Alonso-Blanco *et al.*, 1998), rice (Nair et al., 1995; Tsunematsu *et al.*, 1996, and Cho *et al*., 2010), sunflower (Langar *et al.*, 2003), soybean (Mansur *et al.*, 1996; Choi *et al*., 2010).

In *A. thaliana* RILs are generated from a cross between two different ecotypes. For example, Col and L*er* (Lister and Dean, 1993) and Col-0 and C24 (Törjek *et al.*, 2006). The process involves crossing the two inbred strains, followed by multiple rounds of selfing and single seed decent to create a new inbred line whose genome is a mosaic of the parental genome (Broman, 2005). Several *A. thaliana* RIL populations have been used for QTL mapping and subsequent molecular identification of the responsible genes, including those responsible for seed germination and seed longevity (Clerkx *et al.*, 2004), flowering time (Törjek *et al.*, 2006), light response and flowering time (Balasubramanian *et al.*, 2009).

Broman (2005) stated that a panel of RILs has a number of genetic mapping advantages: each strain can be genotyped once; phenotyping multiple individuals from each strain can reduce measurement, individual, and environmental variability; the break points in RILs are more dense than those that occur in any one meiosis thus greater mapping resolution can be easily achieved.

6.1.4. QTL mapping analysis

Understanding the molecular genetic and biochemical basis of complex traits is facilitated by identification of QTL and their underlying genes. Most genetic studies have focused on dichotomous (rather than continuous traits), which are often due to mutation at a single gene. However, many traits such as flowering time in different *Arabidopsis* (Jonathan *et* al., 2005), osmotic adjustment in rice (Robin *et al*., 2003), etc; are quantitative in nature and are affected by many genes and by environmental factors (Broman, 2001).

In *A. thaliana*, the search for QTL has been carried out in a restricted range of ecotypes using mainly Recombinant Inbred Lines (RILs). There are many QTL studies involving the Col-0 by L*er* RILs, mainly focusing on flowering time (Koornneef *et al*., 1998; Soppe *et al*., 2000; Werner *et al*., 2005) or traits related to flowering such as rosette leaf number (Stratton, 1998) and short integument (Ray *et al*., 1996). Other traits include germination rate (Vander-Schaar *et al*., 1997), floral characters (Juenger *et al*., 2000), inflorescence architecture (Ungerer *et al*., 2002), leaf morphogenesis (Robles *et al*., 2001) and trichome spacing and number (Larkin *et al*., 1996).

However, since Col and L*er* are relatively closely related and would therefore not be expected to show the most extensive polymorphism, other QTL studies have used different ecotypes such as Warschau-1 (Wa-1) by Col-0 RIL to locate powdery mildew disease-resistance loci (Schiff *et al*., 2001) and Bay-0 by Shahdara RIL to locate flowering

genes (Loudet *et al*., 2002). Furthermore, Bay-0 by Shahdara RIL as described by Loudet *et al*., (2002) constitutes the largest population in size available in Arabidopsis. They have analysed the genetic basis of variation in flowering time in Arabidopsis in two different environments and identified QTL precisely with *FRIGIDA* and *FLOWERING LOCUS C* genes. However, they also stated that Bay-0 by Shahdara RIL population represents a powerful tool which is currently being used to undertake the genetic dissection of complex metabolic pathways.

Broman (2001) stated that if two strains show phenotypic differences, despite being raised in a common environment, the investigator should be confident that there is a genetic basis for the difference between the two strains. One of the simplest methods to identify the genes underlying such phenotypic differences is using backcrosses, but the principal is the same for other methods including those using RILs. The statistical approaches used in data analysis for different crosses are the same (Collard *et al*., 2005).

The statistical method logarithm of the odds (to the base 10) (LOD score) (Broman, 2005) is used for linkage analysis in humans, animals, and plant populations. A positive LOD score indicates the presence of linkage, whereas a negative LOD score indicates that linkage is less likely (Morton, 1990). The LOD score analysis is the simplest method to analyse complex family pedigree in order to determine the linkage between Mendelian traits or between trait and a marker or two markers.

The work described in this chapter was aimed at increasing our understanding of the mechanism underlying the Col-killer trait by mapping and ultimately identifying the gene or genes responsible. Gene mapping requires genetic variation for the trait between genetically polymorphic parents, and a means to score the trait in a segregating population. A difficulty with the Col-killer trait in this context is that its expression is restricted to a tetraploid pollen parent, and then only when crossed to a diploid seed parent of either the same ecotype, or one of number of submissive ecotypes (4.2.3.2). Thus any mapping strategy would require the means to both generate a tetraploid population segregating for the trait, and score a potentially large number of segregants for the trait using crosses to a submissive diploid seed parent.

6.2. Results

6.2.1. Strategies for the genetic analysis of the Col-killer trait

Genetic variation for the Col-killer trait that was potentially useful in establishing a mapping strategy was demonstrated in chapter 4. Crosses between maternal Col 2x and Col 4x or L*er* 4x pollen parents revealed dramatic ecotypic differences in the frequency of shrivelled seeds among the progeny (4.2.3.1). The frequency of shrivelled seed in the [Col 2x X Col 4x] and [Col 2x X L*er* 4x] crosses was 89.2% and 6.1%, respectively. Thus, these two ecotypes appeared to have a high degree of genetic variation for the killer trait. Since these ecotypes are also genetically polymorphic, a mapping strategy was developed based on Col-0 and L*er* parents.

As described previously (6.1.3), Recombinant Inbred Lines (RILs) have proved highly effective in mapping genes where genetic variation exists for a trait between two ecotypes of *A. thaliana*. The Lister-Dean RIL population was constructed from Col-0 and L*er* parents and was therefore potentially useful in mapping the Col-killer. Thus a mapping strategy was developed using this genetic resource. Since a diploid pollen parent does not express the killer trait, the approach envisaged was to produce tetraploid derivatives of sufficient 'core' RIL lines to rough map the trait, and score the 4x RILs in crosses to Col 2x. The *A. thaliana* CSS and STAIR lines (6.1.2) was a second genetic resource with potential for mapping the killer trait, since these like the Lister-Dean RILs were developed from Col and L*er* parents. However, as described below only a limited attempt at their use was made in the present work.

6.2.2. The Col-killer trait is expressed in tetraploid Col/L*er* F_1 hybrids

Since mapping of the Col-killer trait was to be conducted using a genetic resource that had hybrid Col and L*er* genomes, it was important to demonstrate that such a hybrid was capable of expressing the trait. To do this, two F_1 hybrids, Col/L*er* 4x and L*er*/Col 4x, were made by crossing 4x L*er* and 4x Col-0 plants in both directions and collecting the resulting seed. F_1 hybrids plants derived from this seed were then used as pollen parents in crosses to 2x Col-0 seed parents and the frequency of plump and shrivelled seed determined in the resulting siliques (Figures 6.1 and 6.2). Control crosses between Col-0 2x seed parents and both original 4x parents from L*er* and Col-0 ecotypes were made at the same time.

The frequency of shrivelled seed in [Col 2x X (Col/L*er* 4x)] and [Col 2x X (L*er*/Col 4x)] crosses was similar: 49.7 % and 49.3 %, respectively (Figure 6.1). The control crosses behaved as expected: the frequency of shrivelled in the [Col 2x X Col 4x] and [Col 2x X L*er* 4x] crosses was 81.1 % and 9.4 %, respectively. This data shows that the combination of both haploid genomes of L*er* and Col-0 within a diploid sperm, are able to disrupt seed development, but at a reduced frequency compared to Col 4x diploid sperm, suggesting that the trait is semi-dominant trait.

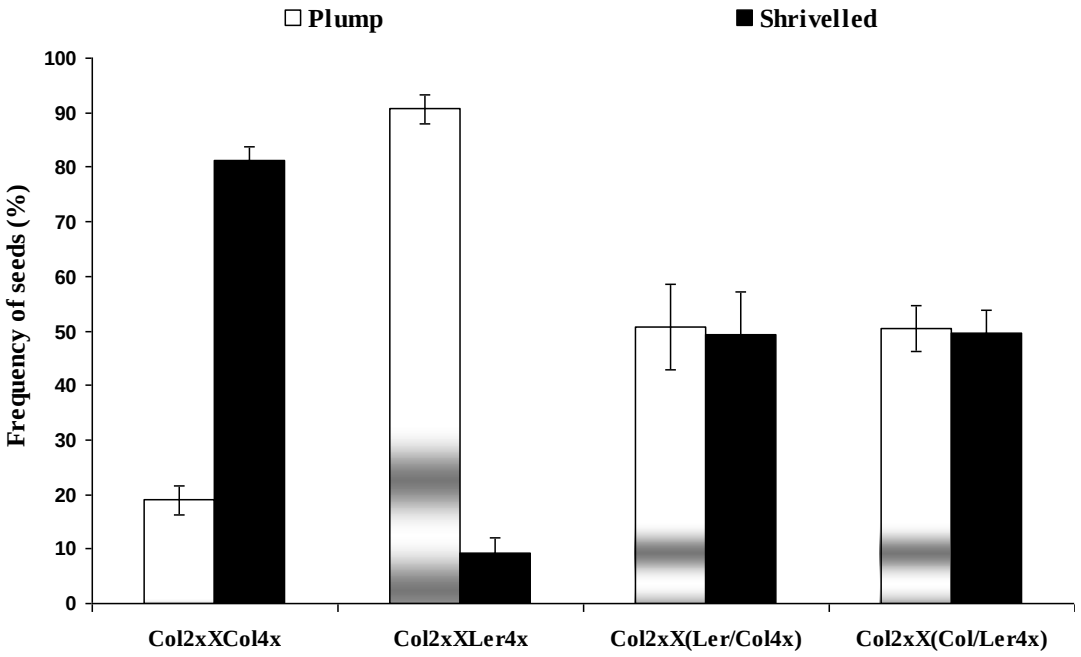


Figure 6.1: Frequency of abnormal seed production due to tetraploid Col/L*er* pollen parents.
Error bars represent the standard error of the mean.

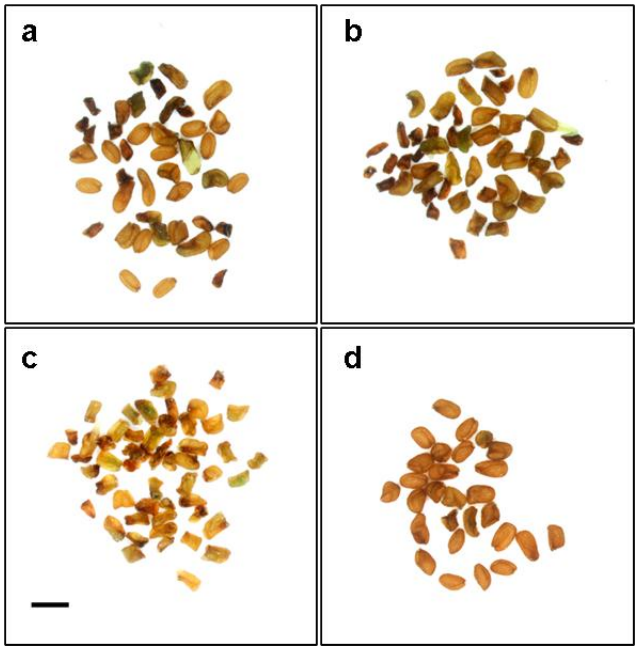


Figure 6.2: Seed produced in crosses using to 4x Col/L*er* pollen parents. (a) Col 2x X (Col /L*er* 4x) (b) Col 2x X (L*er*/Col 4x) (c) Col 2x X Col 4x (d) Col 2x X L*er* 4x. Scale bar = 1mm

6.2.3. Rough mapping of the Col-0 killer trait using CSS and STAIR lines

The aim of this experiment was to identify the chromosome or chromosomes carrying the Col-killer gene(s) using *A. thaliana* (Col/L*er*) plants from both CSS and the Single Recombinant Line (SRL1). The genomes of CSS lines are each composed of four Col chromosomes and a single L*er* chromosome, and differ one from another in which chromosome is substituted. There was no CSS line available for chromosome 1; instead this chromosome is represented by the SRL1, which is mostly Col, with a small segment of L*er* at the bottom of the chromosome. The composition of the lines is shown diagrammatically in Figure 6.3. The rationale in using these lines was that assuming the Col-killer trait resides primarily on only one of the Col chromosomes (1-5), all the 4x CSS lines would cause killing, except for the line in which the Col-killer containing chromosome was substituted with L*er* chromosomes.

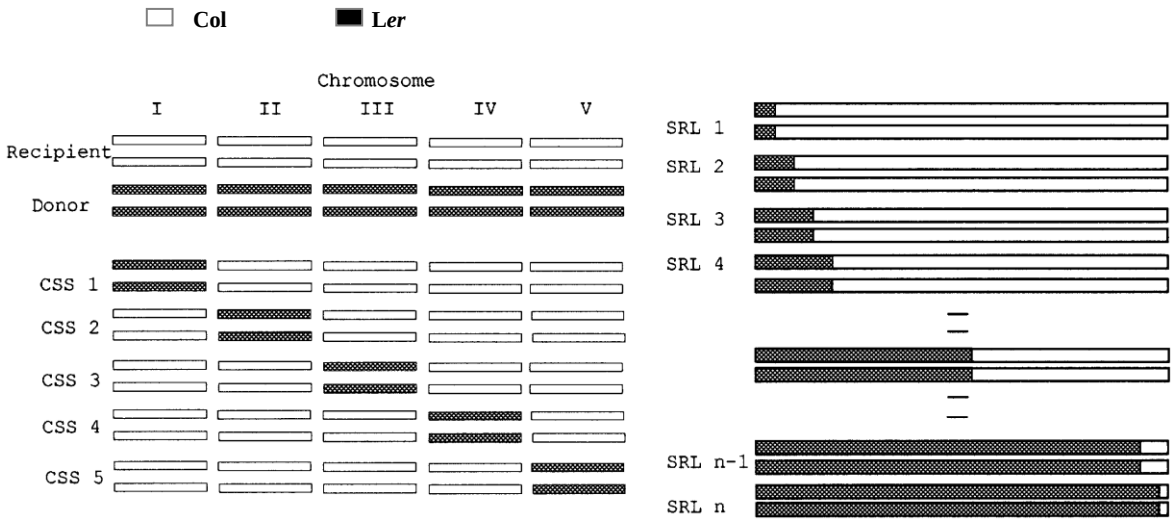


Figure 6.3: Chromosome Substitution Strains (CSS) and Single Recombinant Lines (SRL) making up the Stepped Aligned Inbreed Recombinant Strains (STAIRS) in *A. thaliana*.
Adapted from Koumproglou *et al*., (2002).

6.2.3.1. Generating tetraploid 4x populations from diploid 2x CSS and STAIR (SRL1) plants

Tetraploid derivatives were generated from both CSS (2-5) and SRL1 (chromosome 1) using colchicine. It was important to compare the newly generated plants to their diploids to assess whether the colchicine treatment had resulted in chromosome doubling. Potential tetraploid plants were first identified based on an increase in flower size relative to diploid controls (Figure 6.4). Seed was then collected from these plants and weighed. Plants with large flowers also showed a large increase in mean seed weight compared to the diploid controls (Student t-test, $P < 0.001$) (Figures 6.5 and 6.6). Finally, these plants were tested by karyotyping to confirm a tetraploid chromosome number. An example karyotype is shown in Figure 6.7. All plants with large flowers and seed were successfully confirmed as tetraploid.

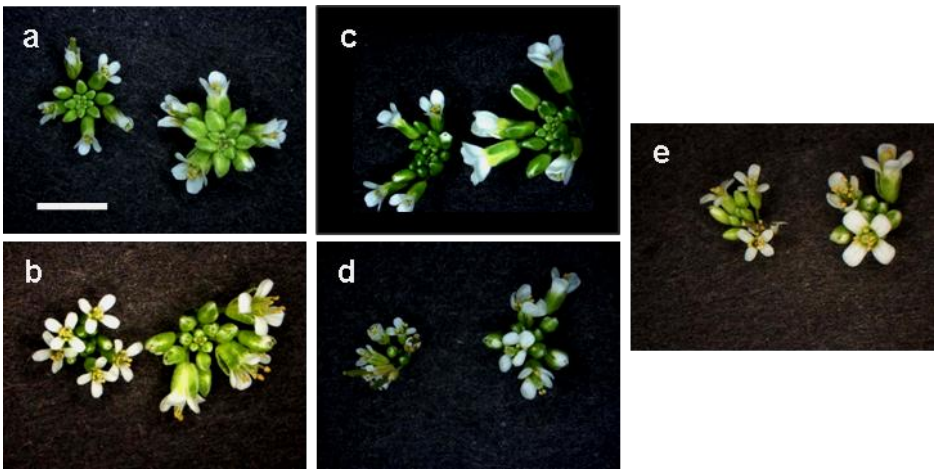


Figure 6.4: Effect of colchicine treatment on the size of both flowers and inflorescences of CSS 2-5 and SRL1 lines. (a) CSS 2 (b) CSS 3 (c) CSS 4 (d) CSS 5 (e) SRL1. The untreated 2x is on the left, and the potential colchicine-induced 4x is on right. Scale bar = 0.5 cm.

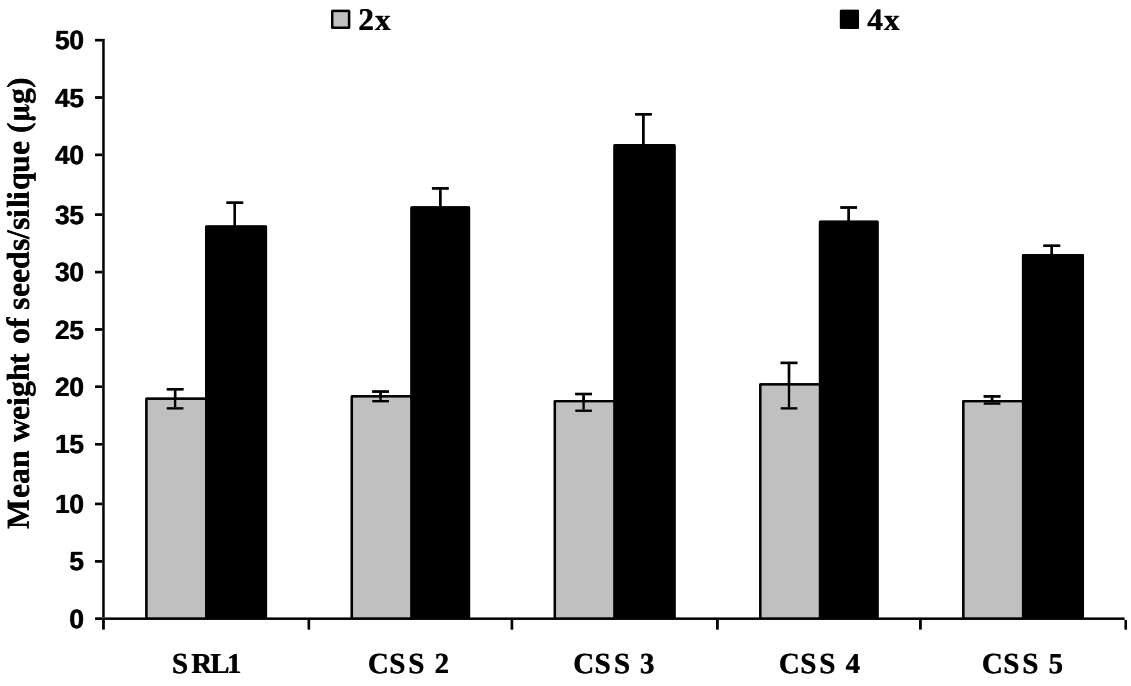


Figure 6.5: Seed weight analysis of potential tetraploid plants derived from SRL1 and CSS lines 2-5.

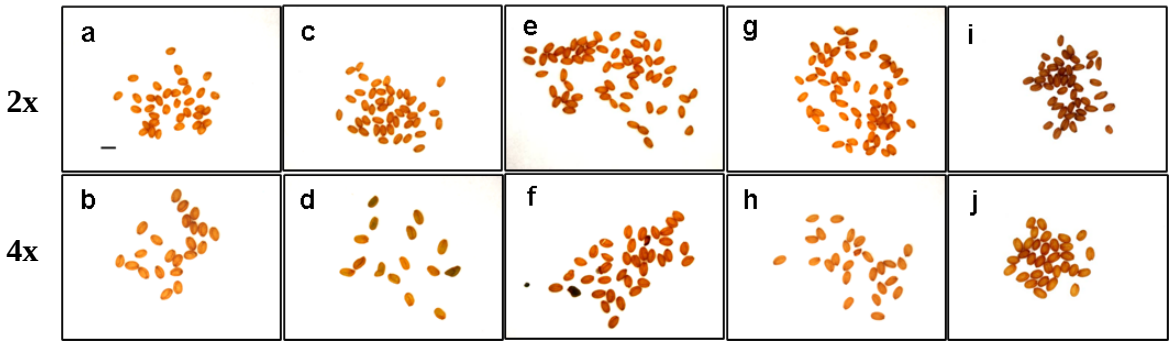


Figure 6.6: Seed produced by (Col/L*er*) CSS and SRL1 lines showing both the diploid and the derived tetraploid.
(a,b) CSS2 (c,d) CSS3 (e,f) CSS4 (g,h) CSS5 (i,j) SRL1. Scale bar = 1mm

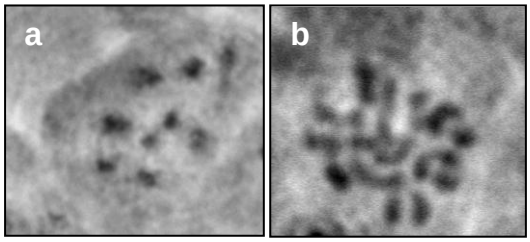


Figure 6.7: Chromosome karyotype of diploid and tetraploid CSS5.
(a) 2x (10 chromosomes); (b) 4x (20 chromosomes)
Magnification = 100x

6.2.3.2. The Col-0 killer trait: one locus or a QTL?

The aim of this experiment was to identify whether the Col-killer trait was carried on a single chromosome or dispersed across multiple chromosomes. Crosses between a Col 2x seed parent and 4x CSS 2-5 and SRL1 lines were performed and impact on seed hybrid development assessed by measuring the frequency of shrivelled seed (Figures 6.8 and 6.9) and the mean seed weight (Figure 6.10). The frequency of shrivelled seed was high in all F_1 progenies, ranging from 67.6% for CSS4 4x to 90.5% for CSS3 4x (Figure 6.8). These compare to control values of 79.5% and 8.2% for [Col 2x X Col 4x] and [Col 2x X L*er* 4x], respectively. This data indicated that based on the frequency of shrivelled seed the tetraploid CSS and SRL lines closely resembled Col 4x in their behaviour in [Col 2x X Col 4x] crosses.

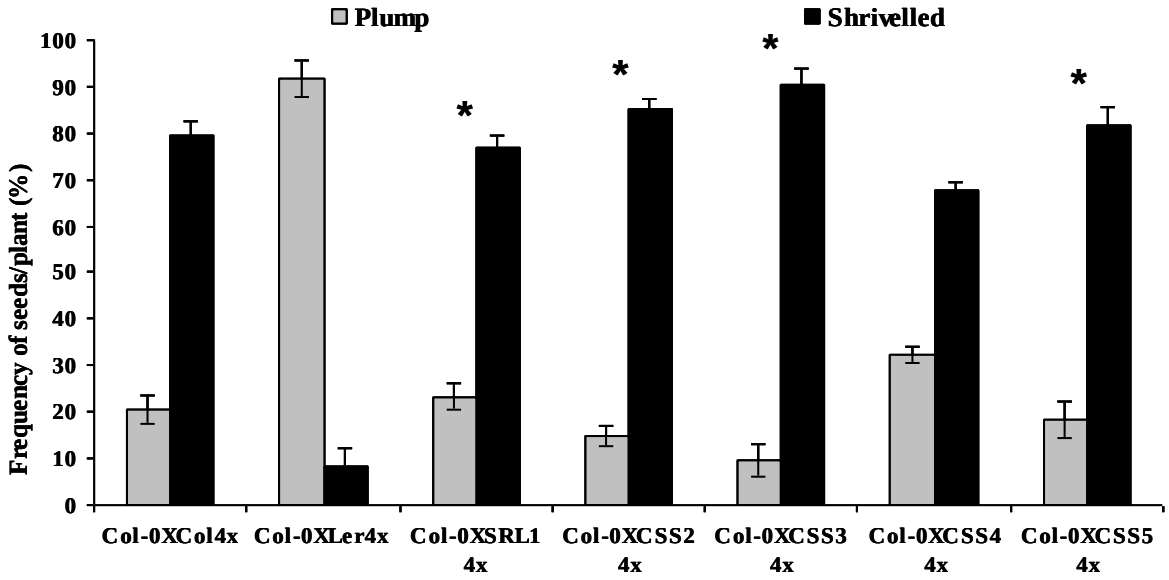


Figure 6.8: Frequency of shrivelled seed in the progeny of crosses between a Col 2x seed parent and tetraploid CSS and SRL1 pollen parents.
The frequency of the plump and shrivelled seeds in the progeny of these crosses was determined and the mean for each cross is given along with the standard error. * Non-significant at P > 0.05 compared to [Col 2x X Col 4x].

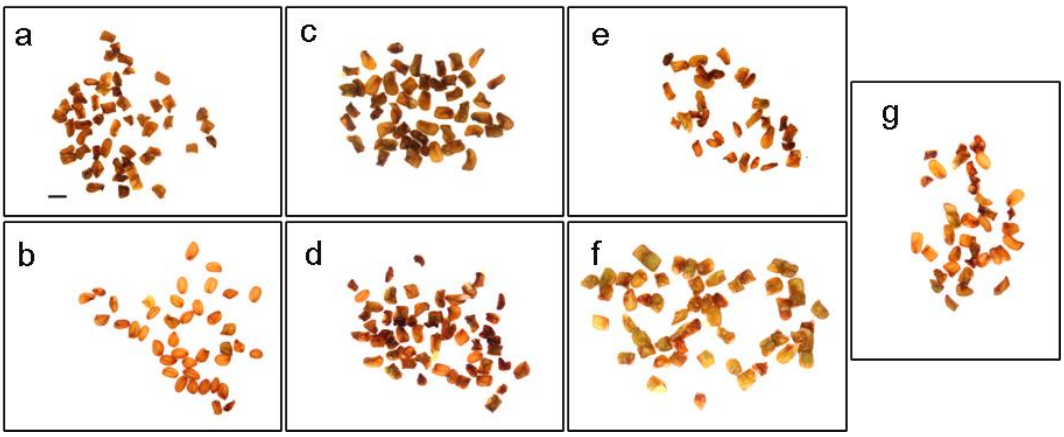


Figure 6.9: Seed produced in crosses between a Col 2x seed parent and tetraploid CSS and a chromosome 1 SRL pollen parents.
(a) Col 2x X Col 4x; (b) Col 2x X L*er* 4x; (c) Col 2x X SRL1 4x; (d) Col 2x X CSS2 4x; (e) Col 2x X CSS3 4x; (f) Col 2x X CSS4 4x; (g) Col 2x X CSS5 4x. Scale bar = 1mm

Analysis of the mean weight of F_1 hybrid seed mirrored the shrivelled seed frequency data (Figure 6.10). The mean seed weight was low in all F_1 progenies, ranging from 11.8 µg for CSS3 4x to 22.2 µg for CSS4 4x. These compare to control values of 19.5 µg and

33.4 µg for [Col 2x X Col 4x] and [Col 2x X L*er* 4x], respectively. Thus the mean seed weight data indicated that all four tetraploid CSS lines and the tetraploid SRL line closely resembled Col 4x in their behaviour in [Col 2x X Col 4x] crosses.

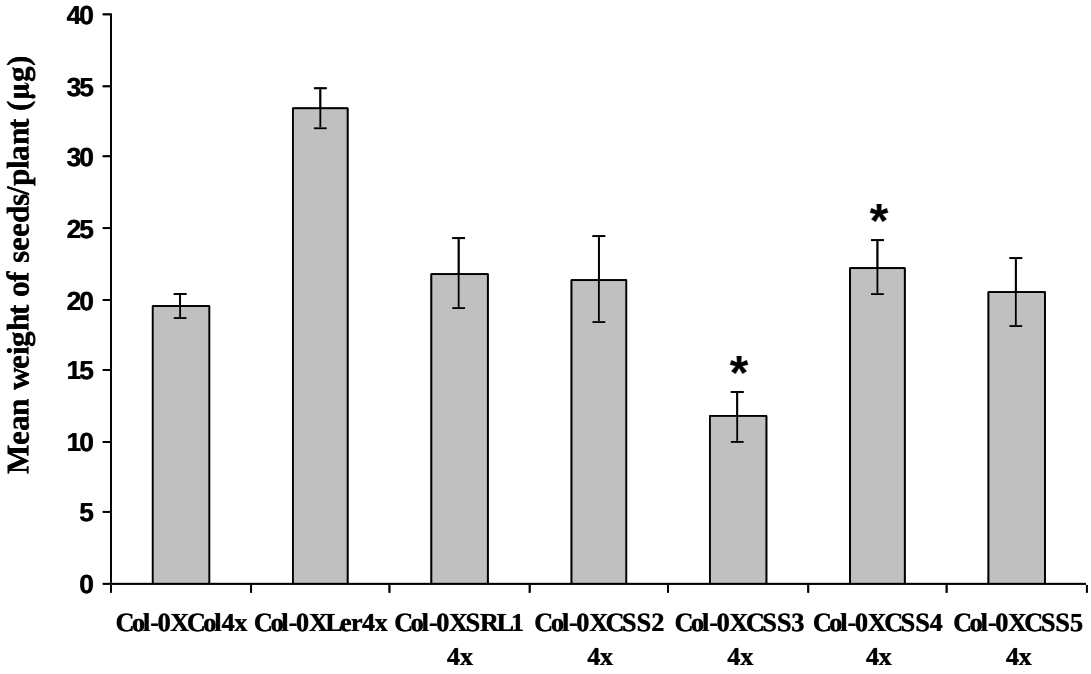


Figure 6.10: Mean weight of seeds / plant in the interploidy crosses between 2x Col and CSS 2-5 and SRL1 *A. thaliana* tetraploid 4x plants.
* Significant at P > 0.05 compared to [Col 2x X Col 4x].

The frequency of shrivelled seed and the mean seed weight data presented above did not identify a single chromosome as carrying the Col-killer trait. This could have indicated that the trait requires the action of multiple genes i.e. a quantitative trait, and that the quantitative trait loci are located on multiple chromosomes, and perhaps interact. A single chromosome substitution could then fail to eliminate Col-killing, as observed above. A second possibility was that the trait is a single locus and located within the small region of Col DNA at the southern of the chromosome 1 SRL. If true, this SRL would retain its ability to cause Col-killing, as observed above. In the absence of a firm foundation from which to extend the use of CSS and STAIR lines in mapping the Col-killer, the alternative of RIL-based mapping was pursued.

6.2.4. Mapping the Col-0 trait using tetraploid Recombinant Inbred Lines (RILs)

A total of 70 Lister- Dean RI lines (Col/L*er*) (Table 2.2) were selected from the core 100 lines available at NASC and used to produce tetraploid derivatives using the colchicine method. The tetraploid RILs were then used as pollen parents in crosses to a Col-0 seed parent and their Col-killing activity scored using shrivelled seed frequency and mean seed weight measures. This data was then analysed using QTL Cartographer software to determine the position and magnitude of any QTL.

6.2.4.1. Generating tetraploid 4x RILs

The process of generating tetraploid derivatives of the RILs was performed essentially as described for the CSS and SRL lines above, but using two concentrations of colchicine (0.25% and 0.125 %) to increase the probability of obtaining tetraploid derivatives, since experience from previous experiments had shown that responsiveness to colchicine was genotype dependent. Potential 4x RIL plants were first identified by their large flower size relative to the untreated diploid (Figure 6.11). Selfed seed was harvested from these plants and the mean seed weight determined (Figure 6.12). Plants with large flowers almost invariably produced seed that was significantly heavier (Student t-test, $P < 0.001$) than diploid seed of that RIL. For example, the mean seed weight for the putative N1911 4x was 36.1 μg compared to 22.0 μg for its diploid. Figure 6.13 compares mature seed of the diploid and derived putative tetraploid for N1911 and a selection of other RILs.

Figure 6.11: Effect of colchicine treatment on the size of both flowers and inflorescences in some RILs
(a) N1911 (b) N1951 (c) N1963 (d) N1970 (e) N1984 (f) N1989. The untreated 2x is on the left, and the potential colchicine-induced 4x is on right. Scale bar = 0.5 cm

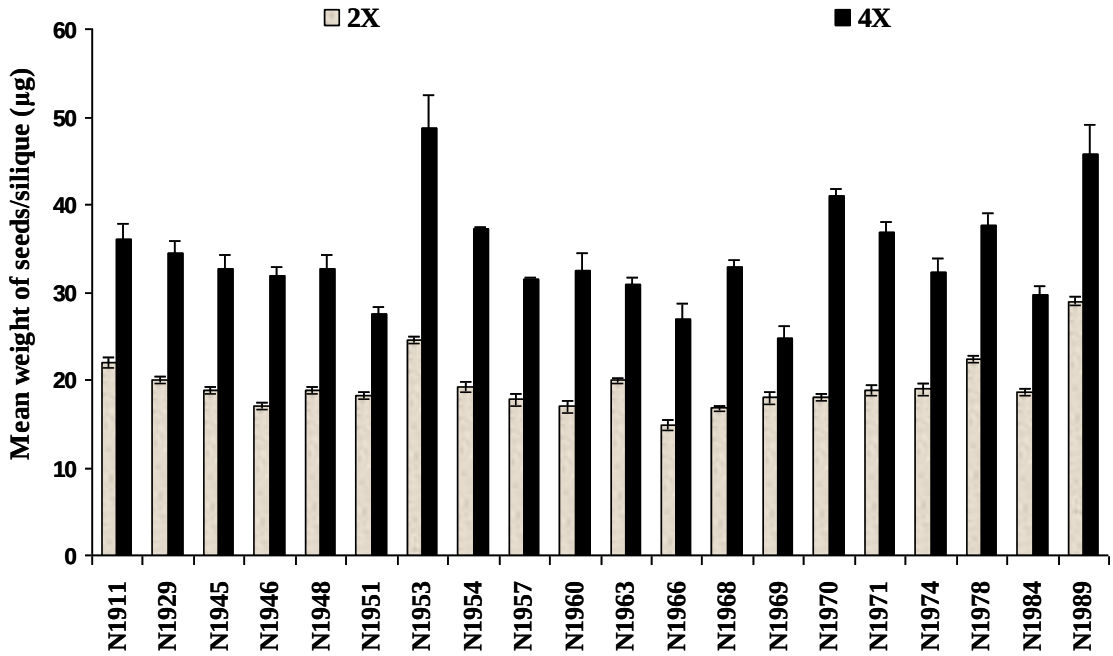


Figure 6.12: Seed weight analysis of potential tetraploid plants derived from selected RILs. The mean weight of seeds/silique is shown for 20 different 2x and their potential 4x derivatives. (Not all data are shown: 20 from total 70 lines) Significant at $P < 0.001$

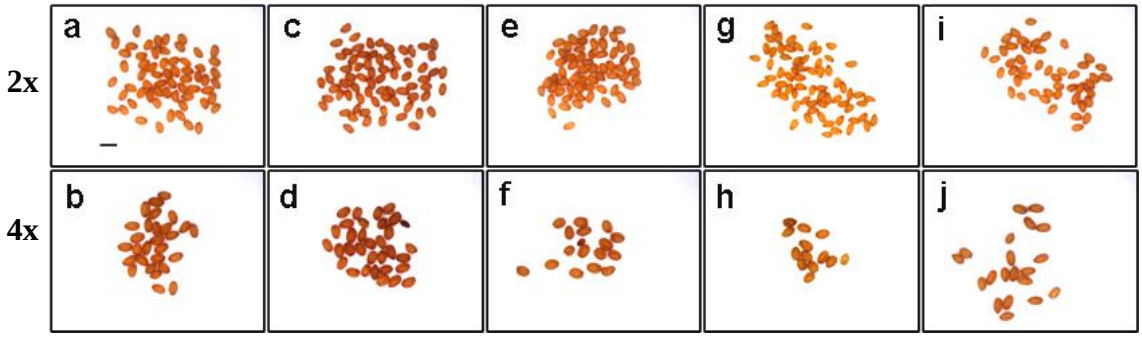


Figure 6.13: Seed produced by a selection of diploid RILs and their potential 4x deriviatives.
(a,b) N1911 (c,d) N1929 (e,f) N1945 (g,h) N1960 (i,j) N1974 . Scale bar = 1mm

Finally, plants with large flowers and a high mean seed weight were karyotyped to confirm a tetraploid chromosome number. An example karyotype is shown in Figure 6.14. All plants with large flowers and a high mean seed weight were confirmed as tetraploid.

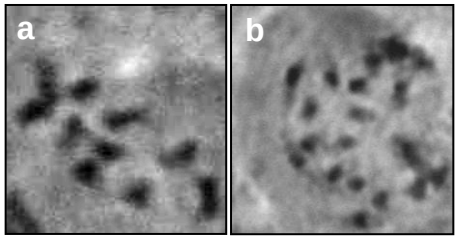


Figure 6.14: Chromosome karyotype in N1990 RIL.
(a) 2x (10 chromosomes) (b) 4x (20 chromosomes) Magnification=100x

6.2.4.2. Mapping the Col-killer trait using tetraploid RILs

A total of 70 tetraploid [Col-0 X L*er*] RIL lines were successfully generated from their respective diploids (Table 2.2). The next step in the mapping procedure was to cross each 4x RIL as a pollen parent to a Col 2x seed parent and estimate % of shrivelled seeds and mean seed weight in each of these crosses (Table 6.1 and Figure 6.15). As described previously, the most reliable measures of cross outcome were shrivelled seed frequency and mean seed weight. In an attempt to produce robust data, both measures were applied to the progeny of the crosses. We then used the software QTL cartographer to identify regions of the genome that are significantly associated with the phenotypes measured.

Table 6.1: Seeds produced from 2x Col-0 and 4x RILs crosses.
The mean weight of seeds/silique and the frequency of shrivelled seeds in [Col 2x X RILs (N1900-N04686) 4x] was determined in each cross with both control crosses [Col 2x X Col 4x] and [Col 2x X L*er* 4x].

Cross	Mean seed weight (ug)	Frequency of shrivelled seeds (%)	Cross	Mean seed weight (ug)	Frequency of shrivelled seeds (%)
Col 2xX Col 4x	18.7	81	**N1949 4x**	17.1	89
Col 2xX L*er* 4x	31.5	29	**N1950 4x**	21.3	78
N1900 4x	25.9	42	**N1951 4x**	30.9	22
N1901 4x	17.1	86	**N1952 4x**	22.1	69
N1903 4x	32.3	25	**N1953 4x**	13.1	85
N1906 4x	13.5	83	**N1954 4x**	24.5	38
N1907 4x	32.1	21	**N1955 4x**	13.4	90
N1909 4x	22.8	68	**N1956 4x**	27.3	33
N1910 4x	30.8	14	**N1957 4x**	20.6	29
N1911 4x	25.8	45	**N1958 4x**	29.6	18
N1912 4x	34.7	14	**N1960 4x**	27.2	47
N1914 4x	24.5	61	**N1961 4x**	26.1	44
N1915 4x	29.2	52	**N1962 4x**	27.7	32
N1916 4x	31.3	26	**N1963 4x**	31.9	35
N1917 4x	22.3	68	**N1966 4x**	32.5	24
N1918 4x	32.4	7	**N1967 4x**	34.1	19
N1919 4x	29.1	38	**N1969 4x**	26.3	44
N1921 4x	22.3	32	**N1970 4x**	19.2	67
N1922 4x	27.9	48	**N1972 4x**	32.4	19
N1923 4x	36.8	3	**N1974 4x**	27.3	0
N1925 4x	14.4	78	**N1978 4x**	19.7	75
N1926 4x	21.3	69	**N1979 4x**	32.4	25
N1927 4x	16.1	76	**N1980 4x**	40.4	4
N1929 4x	27.2	32	**N1982 4x**	35.8	19
N1930 4x	22.2	72	**N1983 4x**	31.2	12
N1931 4x	27.2	35	**N1984 4x**	25.7	19
N1933 4x	31.9	27	**N1985 4x**	32.7	22
N1936 4x	30.1	7	**N1988 4x**	29.3	25
N1937 4x	27.9	50	**N1989 4x**	19.8	92
N1938 4x	26.5	27	**N1990 4x**	32.3	14
N1940 4x	32.5	29	**N1991 4x**	35.8	3
N1942 4x	25.3	3	**N1992 4x**	18.2	83
N1943 4x	35.2	17	**N1993 4x**	30.1	26
N1944 4x	25.4	72	**N1997 4x**	32.3	19
N1945 4x	26.2	47	**N1998 4x**	25.6	25
N1946 4x	29.7	7	**N04686 4x**	39.1	12
N1948 4x	30.2	11			

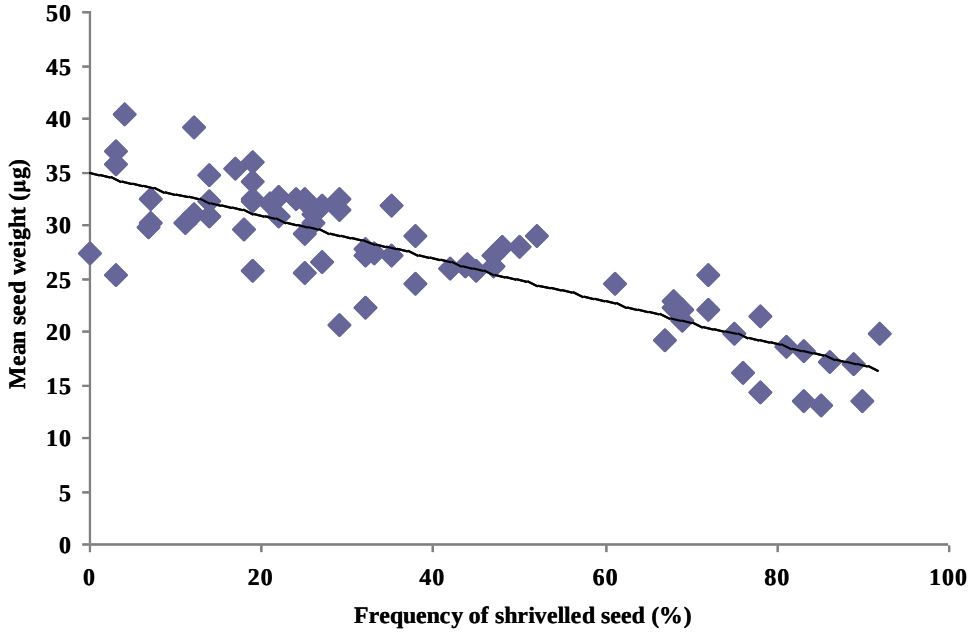


Figure 6.15: Analysis of the linear relationship between mean seed weight and the frequency of shrivelled seeds in the progeny of crosses between a Col 2x pollen parent and tetraploid RILs. Continuous line indicates best fit line.

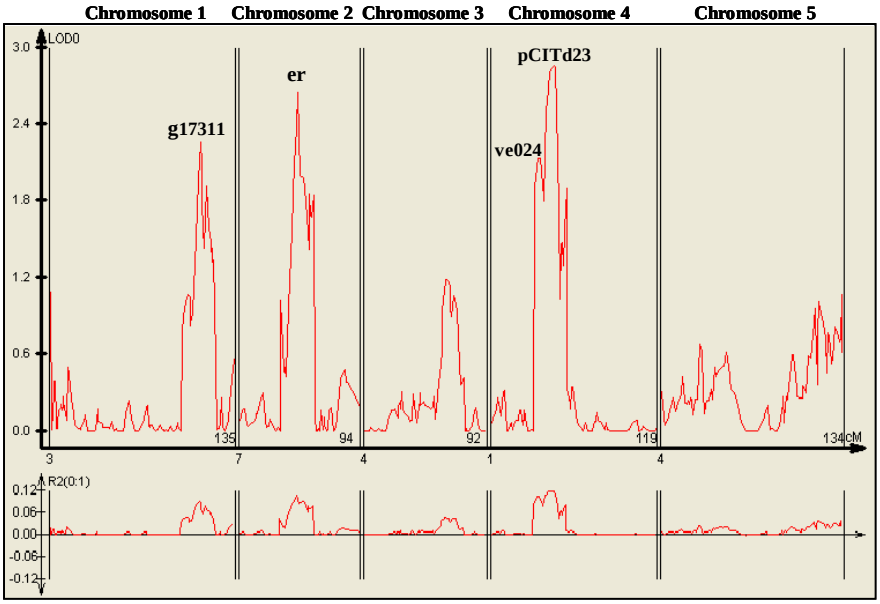
QTL analysis from the mean seed weight measurement as shown in Figure 6.16 of crossing 2x Col-0 (seed parent) with 70 4x RILs (pollen parent) using CIM identified four QTLs that had significant effects in controlling the Col-0 killing trait via segregation data from a number of DNA markers. A LOD score (LOD>2.0) was used as a threshold to determine the presence of Col-0 killing trait. This value was estimated using a permutation analysis with the threshold value for the QTL and determined by 1000-time permutation test (Churchill and Doerge, 1994), to provide a genome-wide 0.05 significance level. The names of markers and locations of the QTL are shown in Table 6.2. A total of 4 QTLs were found across 3 chromosomes of (Col/L*er*) RILs used for identifying the Col-0 killing trait as shown in Figure 6.16.

As displayed in Table 6.2 and Figure 6.16, the first QTL was centred on *g17311* marker on chromosome 1 with a position of (125.4 cM), and a LOD score of ~ 2.25. However, the additive effect of Col-0 allele at this position had a ratio of 5.2, i.e. the QTL effect indicates that the Col-0 allele increases the killing trait by increasing the frequency of shrivelled seeds in the [Col 2x X 4x RIL] crosses. The second QTL was close to the gene *ERECTA* (*ER*) on chromosome 2 (50.0 cM) and had a peak LOD score ~ 2.7. The Col-0 allele increased the killing trait at this position with a ratio of 13.2. There are another two killing QTLs acting additively on chromosome 4, the first was located on *pCITd23* (40.3 cM); while the other QTL was closely located to *ve024* marker (51.9 cM). The

additive effect ratio of Col-0 allele at both positions was 12.7. The Col-killer QTL overlay: 2627.5 kb (*g17311*) on chromosome 1; 5740 kb (*er*) on chromosome 2; 4895 kb (*pCITd23*); and 397.5 kb (*ve024*) on chromosome 4 of the chromosomal DNA. The total length of the *A. thaliana* genome is ~ 125 Mb. Consequently, identification of the Col-killing trait requires further work to reduce the interval size. The QTL contain a total of approximately 2420 genes. Information about the genes underlying the Col-0 killer QTL is shown in Figure 6.17.

Table 6.2: Results of Col-0 killing trait QTL analysis. QTL positions, Markers name, and additive effect of Col-0.

Chromosome	Marker	Position (cM)	Additive effect of Col-0 allele
1	g17311	125.4	5.2
2	er	50	13.2
4	pCITd23 ve024	40.3 51.9	12.7



DNA length underlying each QTL **2627.5 kb** **5740 kb** **4895 kb** **397.5 kb**

Figure 6.16: Chromosomal location analysis of the QTL controlling the killing activity in Col-0 using Col/L*er* RILs. QTL analysis of the killing trait was quantified using the mean seed weight in 70 4x RILs (pollen parents) crossed with 2x Col-0 (seed parents). The peak LOD score was located at *g17311*, *er* and *pCITd23/ve024* intervals on chromosomes 1, 2 and 4 respectively. DNA length underlying QTL Col-killer trait is shown in kilo base (kb).

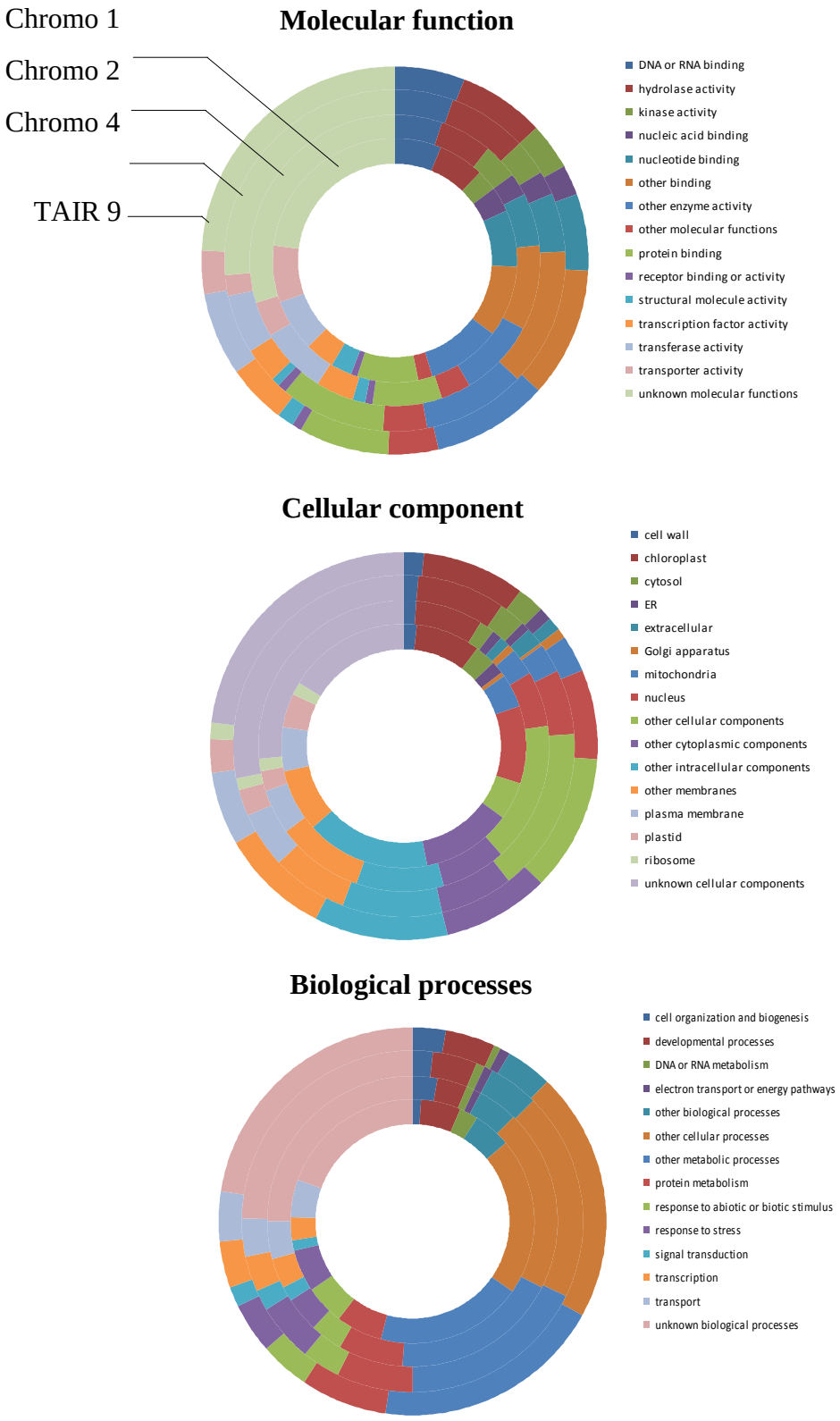


Figure 6.17: Gene ontology within the Col-0 killing QTL.

6.3. Discussion

The analysis of quantitative traits in recent years has widely focused round the use of molecular markers to locate and measure the effects of the individual underlying genes, quantitative trait loci or QTL (Tanksley, 1993; Kearsey and Farquhar, 1998; Kearsey *et al.*, 2003). The primary aim of the work reported in this chapter was to elucidate the QTL of the killer trait caused by the *A. thaliana* Col-0 ecotype. It was important to study one of *A. thaliana* traits that concerned as a hybridisation barrier for seed development, and thereby identify the gene/genes responsible for seed abortion using different tools from molecular genetics resources. However, the identification of QTL of the Col-0 killer trait could pave the way to clone some related specific genes for crop improvement.

6.3.1. STAIRS, CSS and RIL are powerful tools in identifying the QTL of Col-0 killer trait

The STAIRS have several advantages in locating a trait or several traits on a chromosome. However, they provide a route to zoom in rapidly on a chromosome region of interest so facilitating fine mapping. They benefit from several features: single recombinants are more frequent and therefore cheaper to produce, the recombination position is easily identified using them, there is no fear of complications due to secondary substitution, and very small differential regions can be achieved with complete genome coverage (Koumproglou *et al.*, 2002). In this part of the research both CSS lines (2-5) and the STAIRS (SRL1) were crossed as tetraploid pollen parents with maternal Col 2x to identify the QTL of the Col-0 killer trait. The data from both frequency of shrivelled seeds and mean seed weight measurements in Figure 6.4 showed that the diploid lines of both STAIR (SRL1) and CSS (2-5) were successfully transformed into tetraploids after subjecting these diploid strains to colchicine treatment. The experiment was carried out to locate the Col-0 killer trait on the 5 *A. thaliana* chromosomes. However, the mean seed weight measurement was more robust in identifying the QTL location on chromosomes 1, 2 and 4, thus the data showed slightly higher mean seed weight values for SRL1, CSS 2 and CSS 4 than the control [Col 2x X Col 4x] (Figures 6.8 and 6.10). Koumproglou *et al.*, (2002) suggested that the set of whole chromosome substitution plus STAIRS will allow rapid identification of a gene first to a chromosome and then to a particular region defined by known and sequence linked markers. Furthermore, they recommended using STAIRS in order to achieve finer mapping.

The recombinant inbred lines (RILs) used in this study provided a potentially powerful tool for the genetic analysis of quantitative traits and have been a major impetus to quantitative genetical research and breeding. Using both the frequency of shrivelled seeds and mean seed weight measurements was necessary to identify new tetraploid (Col/L*er*) RIL populations. The tetraploid 4x RILs showed higher mean seed weight and larger structures (e.g. flowers, inflorescence, etc) as demonstrated in both Figures 6.12 and 6.13. However, the tetraploidy was confirmed by subjecting young inflorescence meristems to karyotyping (Figure 6.14). Taking advantage of RILs derived from crosses between both ecotypes Col-0 and L*er* (Lister and Dean, 1993), the QTL killer trait of Col-0 was mapped to QTL located on chromosomes 1, 2 and 4.

6.3.1.1. Advantages of RIL in QTL mapping

RILs are an important resource for investigation and genetic mapping for quantitative and qualitative characters in many plant species. They also represent an immortal segregating population allowing the inherent environmental error to be reduced by replication and providing resourceful system for QTL analysis due to their high degree of homozygosity (Borevitz *et al.*, 2002; Törjek *et al.*, 2006). An important advantage of using the (Col/L*er*) RILs is that after genotyping, the same population can be used for the mapping of any number of traits for which the parents differ in order to reduce the costs and effort required to identify positions of QTL (Törjek *et al*., 2006).

The accuracy of QTL mapping is still a problem because it depends on several factors such as the heritability of the trait, the number of QTL involved, the distribution of the QTL over the genome, the number of the marker loci and their distribution over the genome and the population size (Bandaranayake *et al.*, 2004; Knapp *et al.*, 1990). However, the precision of QTL position depends more on population size than the number of markers and no remarkable expansion in accuracy is achieved with more than five well markers for each chromosome (Kearsey and Pooni, 1998). Moreover, it is advisable to use a mapping population of relatively large size and QTL of high heritabilities for consistent estimation of QTL effect.

As demonstrated in chapter 4 the killing activity caused by the paternal 4x Col-0 ecotype played a major role in *A. thaliana* hybridisation barriers when crossed with other diploid ecotypes (4.2.3.2). However, the results showed variation in F_1 lethality from both maternal Col-0 and L*er* ecotypes when crossed with Col 4x pollen parent, thus indicating a major difference in their resistance activity toward paternal Col 4x. The cross between [Col

2x X Col 4x] showed higher levels of shrivelled seeds (89.2 %) compared to [L*er* 2x X Col 4x] cross (41.8 %). In the present work, a number of crosses was performed between Col 2x as seed parent with the newly generated 4x (Col/L*er*) 70 RILs as pollen parents and recorded different levels of lethality in [Col 2x X RIL (Col/L*er*) 4x] to identify the location of the QTL.

Perez-Vega *et al.*, (2010) stated that in a regular genetic analysis, QTLs can be identified only where the parents show apparent differences. However, it has been known previously by (Dilkes *et al*., 2008) that apparent differences can be demonstrated in the crosses between [Col2x X Col 4x] that result in dead seeds while [L*er* 2x X Col 4x] showed a higher frequency of viable seeds. The mean seed weight of the [Col 2x X RIL 4x] populations was significantly variable in their measurement analysis compared to either [Col 2x X Col 4x] or [L*er* 2x X Col 4x] and such difference could be due to the segregation distortion. The data in Figure 6.15 showed a relation between the frequency of shrivelled seeds and mean seed weight measurements in the crosses between Col 2x maternal side with 70 tetraploid paternal 4x RILs which has an advantage in the statistical determination of QTL underlying the Col-0 killing trait. The statistical analysis using CIM showed that the QTL was significantly associated with the Col-0 killer trait. The marker defining this QTL was *g17311* (chromosome 1), *er* (chromosome 2), and both *pCITd23* and *ve024* (chromosome 4) (Table 6.2 and Figure 6.16). This information provides a valuable starting point for map-based cloning of the genes underlying this trait.

These QTL overlay 2627.5 kb for *g17311* in chromosome 1, 5740 kb for *er* in chromosome 2, and 4895 kb, 397.5 kb for both *pCITd23* and *ve024* respectively in chromosome 4 of chromosomal DNA; however, they contain a total of approximately 2420 genes. These regions are clearly still large, and further work is required to reduce the interval size before identifying the Col-0 killing is possible.

Similarly, many recent studies have been applied to *A. thaliana* plant in order to identify QTL using different RIL populations. For example, McKay *et al.*, (2008) took advantage from using 346 RIL developed from a cross between Kas-1 and Tsu-1 for mapping QTL underlying natural variation and for dissecting the genetic basis of water-use efficiency differences. Also, Lisec *et al*., (2009) identified QTL responsible for heterotic metabolite in *A. thaliana* using 369 RIL from a cross between two mapping populations Col-0 and C24 ecotypes. Recenly Pèrez-Vega *et al*., (2010) identified QTL controlling phonological traits in common bean (*Phaseolus vulgaris* L.) using 104 RIL derived from an inter-gene pool cross between Xana and Cornell 49242. The traits included such as days

to the start of flowering, days to end of flowering, days to harvest as green pod, and days to maturity, seed size traits such as seed length, seed height, seed weight, and seed width, and seed quality traits such as water absorption, and coat proportion. They also showed that these QTL have high additive effects and greater explained variance, and are more consistently detected across different environments. In maize (*Zea mays* L.), (Barriere *et al.*, 2010) also identified QTLs for cell wall digestibility traits such as increasing starch content, lignin content, dry matter content, yield, and cell wall quality using 240 RILs developed from early maize hybrid in a cross between F838 and F286.

In other crop plants such as rice, Cho *et al*., (2010) identified 49 QTLs associated with six physical traits of cooked rice and seven chemical properties of rice grain using 164 RIL derived from a cross between Milyang23 and Gihobyeo. They also showed that two from these QTL with the highest LOD scores provide a valuable starting point for positional cloning of genes underlying these QTL.

6.4. Conclusion

In summary, the present study reports the identification of QTL for the killer trait caused by the *A. thaliana* Col-0 ecotype. This kind of postzygotic hybridisation barrier controls the lethality in the F_1 progenies from the crosses between diploid and tetraploid individuals of the same genotype. The data presented above demonstrated the usefulness of both STAIRS and RILs for mapping this type of trait in *A. thaliana*. The large set of tetraploid RIL populations used in this project could provide an excellent resource for future resolution of the Col-0 killing QTL Identification of QTL of the Col-0 killer trait would help in cloning some related specific genes for crop improvement.

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Chapter 7

7. General Discussion

The aim of the work described in this thesis was to investigate the nature of hybridisation barriers that occur in interploidy crosses between different *A. thaliana* ecotypes. The postzygotic hybridisation barriers restrict gene flow and isolate populations from one another. In interploidy, intra- and interecotype crosses of *A. thaliana*, the maternal to paternal genomic ratio (2m:1p) in the endosperm is important in determining the outcome of the cross as described by Scott *et al*., (1998a). Also, wide crosses and interecotype hybridisations as shown in Chapter 5, have been used to investigate the genetic basis of complex traits that differentiate varieties within a species in several plant families (Bradshaw *et al.*, 1995; Eubanks, 1997; Lin and Ritland, 1997; Nasrallah *et al.*, 2000).

7.1. Postzygotic hybridisation barriers and its consequences for *Arabidopsis* crosses

A variety of mechanisms minimize gene flow between species and contribute to their reproductive isolation (Rieseberg and Carney, 1998; Rieseberg and Blackman, 2010). One class of mechanism termed a postzygotic hybridisation barrier acts within the seed following fertilisation and causes seed death either through failure in endosperm or embryo development (Haig and Westoby, 1991; Coyne and Orr, 1998; Rieseberg and Carney, 1998; Bushell *et al.*, 2003) (Figure 1.1). In interspecific crosses, seed abortion could be attributed to different factors such as: negative interaction between products of evolutionarily diverged gene sequences, widespread changes to genome structure or gene expression (Rieseberg and Carney, 1998; Bushell *et al.*, 2003), and imbalances between the effects of uniparentally expressed genes (genomic imprinting) (Haig and Westoby, 1991), either because of ploidy imbalance or divergence in gene expression patterns (Bushell *et al*., 2003). A potent postzygotic hybridisation barrier in many species occurs when individuals of different ploidy e.g. diploids and tetraploids, are crossed. The failure of such crosses to produce the expected triploid progeny led to this barrier being termed triploid block. This lethality arises in the absence of any allelic diversity due to the difference in the dosage of genomes provided by the parents. The strength of this reproductive isolation

can vary within species and between individuals of the same species. However, the genetic basis of this barrier and the source of variation within and between species remain poorly understood.

The work reported in Chapter 4 represents a preliminary investigation into the genetic mechanisms responsible for variation in the lethality of interploidy crosses in *A. thaliana*. The objective was to improve our understanding of this type of reproductive isolation barrier, and ultimately identify the genes underpinning the phenomenon. In contrast to many other species, *A. thaliana* apparently did not display triploid block, although crosses between diploid and hexaploid plants did result in tetraploid block (Scott *et al*., 1998a). *A. thaliana* would appear therefore a poor choice for studying ploidy based postzygotic barriers. However, Dilkes *et al.*, (2008) showed that for Col-0 [2x X 4x] resulted in very high levels of seed lethality (Figure 4.1), although the reciprocal [4x X 2x] remained non-abortive. Thus Col-0 possessed what can be described as an asymmetric paternal-only postzygotic hybridisation barrier. Interestingly, Redei (1964) had much earlier described a similar outcome for reciprocal crosses between an *A. thaliana* diploid 'genotype' 'W' and its autotetraploid 'K-16'. Redei confirmed the 'W' genotype as the Columbia ecotype in a personal communication to R. Scott, University of Bath.

The possession by Col-0 of such an extreme asymmetric postzygotic hybridisation barrier, or 'Col-0 syndrome', presented an opportunity to utilize *A. thaliana* to investigate one important mechanism of reproductive isolation. Chapter 4 first addressed the question of which of the two behaviors - no triploid block or paternal-only triploid block - or some other permutation, is most representative of the *A. thaliana* species. The experimental approach was to test the crossing behaviour of a total of 14 ecotypes and to use meiotic mutants to investigate aspects of the Col paternal only triploid block.

The analyses presented in section 4.2.2 showed 1) that *A. thaliana* as a species responds to moderate maternal genomic excess (4m:1p) in the endosperm by producing relatively small but viable seed i.e. there is no maternal triploid block, 2) the majority of ecotypes do not possess paternal triploid block either, although Col-0 is not unique since RLD was discovered to also have a potent paternal triploid block. Thus genetic variation for triploid block occurs in *A. thaliana*, but a larger number of ecotypes should be tested before drawing firm conclusions about prevalence.

Why Col-0 and RLD should have a very similar type of triploid block was puzzling. The question was therefore addressed as to whether the mechanism responsible for the behaviour in the two ecotypes had a common underlying cause (4.2.4.1). Using 4x

Col-0/RLD F_1 hybrids as pollen parents in [2x X 4x] crosses caused abnormal seed development at the roughly the same frequency as the original 4x parents strongly suggesting that the RLD and Col-0 syndromes are underpinned by a common genetic mechanism. Despite extensive investigation nothing connecting the ancestry of the two ecotypes could be found; we cannot say therefore whether their triploid block behaviour arose in a common ancestor, or independently.

Chapter 4 also describes an attempt to better understand mechanistic basis the Col-0 syndrome. Two scenarios were considered: 2x Col-0 is a 'weak' mother or 4x Col-0 is a 'strong' father. Crossing experiments (4.2.3) provided little support for the weak mother idea, but there was evidence that 4x Col-0 can behave as a strong father: crosses to 4 ecotypes (C24, Per, Cvi and RLD) resulted in high levels (>70%) of abnormal seed, and a further 2 ecotypes (L*er* and Stw) produced moderate levels (>40%) of abnormal seed. However, 50% (7 from 14) of the ecotypes tested produced very low levels of abnormal seed. Thus, 4x Col-0 is not a universally aggressive pollen parent, but is highly aggressive when paired with certain ecotypes.

The pairing of certain *A. thaliana* ecotypes has recently been shown to also result in hybrid necrosis (Reviewed in Reiseberg and Blackman, 2010; see also Table 1.1). For example, Bomblies *et al*. (2007) showed that an allele of an NB-LRR gene family member (*DANGEROUS MIX* 1 (DM1)) triggers necrosis when combined with an allele from a second locus (DM2) from another accession. Similarly, (Bikard *et al.*, 2009) showed that seed development is arrested in proportion of F_1 progeny resulting from a cross between Columbia-0 (Col) and Cape Verde Island (Cvi) ecotypes due to ecotype-specific differential silencing of two *HISTIDINOL-PHOSPHATE AMINO-TRANSFERASE* genes, *HPA1* and *HPA2*. It is possible therefore that the ability of 4x Col-0 to lethally disrupt seed development when paired with certain ecotypes but not others has a similar basis.

However, the most challenging feature of the Col-0 syndrome its asymmetry. Any explanation of the syndrome must account for this asymmetry, and in particular that 1) a diploid sperm is disruptive to seed development in a [2x X 4x] cross but not in a [4x X 4x] cross, 2) a haploid sperm is not disruptive, and 3) diploid maternal gametes in Col-0 do not maternalize seeds more than those of other ecotypes in [4x X 2x] crosses.

There are various potential ways in which asymmetry could arise more or less directly upon polyploid formation including genomic rearrangements, epigenetic alterations to DNA and changes in gene expression patterns (Song *et al*., 1995; Weiss and Maluszynska, 2000; Comai *et al*., 2000; Kashkush *et al*., 2002; Osborne *et al*., 2003). An

attractive hypothesis is that the genomic changes that occur when Col-0 becomes tetraploid could have different consequences for maternal and paternal gametes, and therefore explain the behaviour of diploid gametes in Col-0 (and RLD). Mega- and micro-gametogenesis are different in a several ways, including the number of mitoses prior to gamete formation (Scott and Spielman, 2006), the extent of chromatin condensation (Valencia, 1982), development of the surrounding sporophytic tissues (Grossniklaus and Schneitz, 1998; Simao *et al.,* 2007), molecular and genetic basis of cell specification and differentiation (Grossniklaus and Schneitz, 1998). It is possible therefore that the polyploidisation–induced alterations to DNA structure could impact the two genders of gametes differently. What is observed is that 4x Col plants produce benign maternal gametes but paternal gametes that cause massive, lethal, endosperm over-proliferation. The paternal genomes in effect behave as if derived from a hexaploid pollen parent.

Lethal endosperm over-proliferation in [2x X 6x] crosses can be explained by the supernumerary paternal chromosomes supplying an excess of genes that promote endosperm proliferation (Scott and Spielman, 2006). How does a diploid sperm from a 4x Col-0 plant apparently achieve the same level of growth promoter excess as a triploid sperm from other ecotypes? Clearly, this must be achieved without access to additional physical copies of paternally derived growth promoting genes. There are at least two potential sources of extra growth promoting genes. The first is virtual – the paternally-derived growth promoting genes are expressed at a higher level or for longer during endosperm development. This might be achieved by an epigenetic change in the paternal genome, due to the polyploidisation event, that enables the paternal chromatin to resist attempts by the seed parent to reduce paternal expression. Furthermore, it is well known that for many genes expression from the paternal genome is absent or very low during early seed development (Vielle-Calzada *et al.*, 2000). The silencing of the paternal genome is probably related to the tight packaging of sperm chromatin involving specific histones (Ueda and Tanaka, 1995; Xu *et al.*, 1999) or changes in methylation levels of sperm DNA as compared with DNA in the vegetative nucleus (Oakeley *et al.,* 1997). Male-specific polyploidization-induced changes to chromatin packing or genome methylation could enable earlier or higher levels of paternal genome expression following fertilisation leading to endosperm over-growth.

A second source of extra growth promoting genes is the maternal genome. Growth promoting genes are hypothesized to be silent within the maternally-derived genome of the endosperm (Scott *et al.,* 1998a). A possibility is that the Col-0 derived paternal genome is

able to express factors that relieve maternal silencing of these genes. Similarly, paternal repression of maternally-expressed growth inhibitor genes such as *MEDEA* (Grossniklaus *et al.*, 1998) would have the same effect.

Chapter 4 (4.3.4.) also addressed the interesting question of whether the Col-0 syndrome arises only in gametes derived from tetraploids or also appears when the genomes of diploid Col-0 plants fail to undergo meiotic reduction. Experiments with the *A. thaliana* (Col-0) mutants *Atps-1* and *jas-3* (d'Erfurth *et al.*, 2008; Erilova *et al*., 2009), which produce unreduced male gametes, provided some useful insights. Both mutants caused triploid block when used as pollen parents with a 2x Col-0 seed parent indicating that diploid sperm formed by both non-reduction and chromosome-doubling in a tetraploid are similarly disruptive to endosperm development. The data also showed that the disruptive activity arises very rapidly in *Atps-1,* since there are only two mitoses between meiosis II, when non-reduction occurs, and fertilisation.

7.2. The Tsu-1 maternal modifier of Col-killing

The work presented in Chapter 4 revealed that whilst certain ecotypes were highly susceptible to 4x Col-0 killing, others such a Tsu-1 were highly resistant (4.2.1.). Dilkes *et al*., (2008) had previously shown that L*er* offers some resistance to Col-0 killing, and had used this to identify a maternal modifier of Col-killing. We were therefore interested in identifying additional and perhaps more potent maternal modifiers from Tsu-1 since this ecotype had much higher levels of Col-killing resistance than L*er*. This work is described in Chapter 5.

The failure of seed development in Col-0 [2x X 4x] crosses is associated with massive endosperm over-proliferation, both peripheral and chalazal, and a failure to undergo cellularisation (Dilkes *et al*., 2008). However, the *ttg2* maternal modifier identified in L*er* rescues lethality by promoting cellularisation, but has little effect on reduce overall endosperm size. The Tsu-1 modifier was therefore investigated to determine whether a similar mechanism operated. Diagnostic crosses showed like L*er* the Tsu-1 modifier did not markedly reduce the size of the endosperm, but promoted successful endosperm cellularisation.

To gain a better understanding of how modifiers function, an attempt was made in the present work to map and the Tsu-1 modifier and identify the underlying gene or genes. A backcross strategy was adopted and was successfully progressed to the $BC3F_3$ (Figure 5.9). The Tsu-1 modifier appears to be a dominant trait, which simplified the mapping

procedure. The approach adopted was to produce two backcross populations: one with the Tsu-1 allele of the modifier introgressed into the Col-0 background, and the other with the Col-0 allele of the modifier introgressed into Tsu-1. This would generate a 'rescuing' or 4x Col-0 resistant line composed of mostly Col-0 DNA and a none-rescuing line composed Tsu-1 DNA. Analysis using a small number of molecular markers spread across the 5 *A. thaliana* chromosomes suggested that the approach progressing as expected. The lines would then be used to either map the modifier locus using a conventional marker approach, or alternatively to locate the modifier locus by DNA sequencing.

7.3. QTL mapping in plant breeding

The work described in Chapter 6 was aimed at increasing our understanding of the mechanism underlying the Col-killer trait by mapping and ultimately identifying the gene or genes responsible. The dramatically different behaviour of tetraploid Col-0 and L*er* pollen parents in crosses to a Col-0 seed parent mapping suggested a mapping strategy based on Col and L*er* parents. Recombinant Inbred Lines (RILs) have proved highly effective in mapping genes where genetic variation exists for a trait between two ecotypes of *A. thaliana*. The Lister-Dean RIL population was constructed from Col and L*er* parents and was therefore potentially useful in mapping the Col-killer. Thus a mapping strategy was developed using this genetic resource. Since a diploid pollen parent does not express the killer trait, the approach envisaged was to produce tetraploid derivatives of sufficient 'core' RIL lines to rough map the trait, and score the 4x RILs in crosses to Col 2x. The approach identified 4 QTL on three different chromosomes (one QTL in both 1 and 2 chromosomes, and 2 QTL on chromosome 4), which accounted for a large proportion of the Col-0 killing activity. These QTL overlay a physical distance 2627.5 kb, 5740 kb and 4895 kb, 397.5 kb respectively of chromosomal DNA, and contain a total of approximately 2420 genes. These regions are clearly still large, and further work is required to reduce the interval size before identifying the Col-0 killing is possible.

Quantitative genetic approaches have offered significant insights into phenotypic evolution. Most ecologically important traits are quantitative, exhibiting a continuous range of phenotypic variations. For example, plants in natural populations often show quantitative variation in their resistance to damage by natural enemies (Weinig *et al.*, 2003). The quantitative traits in different organisms including plants and animals are controlled by a number of genes that shows continuous variation in progeny.

QTL mapping has been used by many biologists to identify genes with the assistance of DNA molecular markers. The use of DNA markers in plant breeding has opened a new agricultural area called molecular breeding (Rafalski and Tingey, 1993). Also, DNA markers will play an important role in enhancing global food production by improving the efficiency of conventional plant breeding programs (Ortiz, 1998; Kasha, 1999). There are many factors that influence the detection of QTLs segregation in a population (Asins, 2002). The main ones are genetic properties of QTL, which control the traits, environmental effects, population size, and experimental error. However, QTL mapping studies should be independently confirmed or verified due to the previous factors described (Lander and Kruglyak, 1995). Furthermore, some modern studies proposed that QTL effects and positions should be evaluated in independent populations, because QTL mapping is based on typical population sizes in a low power of QTL detection and a large bias of QTL effects (Utz *et al.*, 2000). Unfortunately, due to constraints such as lack of research funding and time, and possibly a lack of understanding of the need to confirm results, QTL mapping studies are rarely confirmed. Most QTL analysis in plants involves populations derived from pure lines as discussed in Chapter 6 and several approaches have been developed to associate QTL with molecular markers in such populations (Kearsey and Pooni, 1996). In some plants e.g. autogamous plants, QTL mapping studies mainly make use of F_2 or backcross progenies because they are the easiest and earliest to obtain (Vanooijen, 1992; Asins, 2002).

In QTL plant breeding studies, some authors suggested that F_2 is better than a backcross since QTL with recessive alleles in a recurrent backcrossed parent could not be detected, and when dominance is present QTL gives bias estimates of the effects because additive and dominant effects are completely confounded in this design (Carbonell *et al.*, 1993). They also found that the degree of dominance in F_2 progenies can be estimated, but there are two inconveniences of F_2 and backcross populations: the genotype cannot be replicated as in double haploids (DHs) or RILs, and epistatic interactions could hardly be studied. In fact, the concepts for detecting QTL were developed more than 75 years ago (Sax, 1923). In recent years the availability of DNA markers and powerful biometric methods has led to considerable progress in QTL mapping in plants. But due to rapid developments in marker technology, statistical methodology for identifying quantitative trait loci (QTLs) and the terminology used by molecular biologists, the utility of DNA markers in plant breeding may not be clearly understood by non-molecular biologists (Collard *et al*., 2005).

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